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Folate production by Propionic acid bacteria

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Tiivistelmä – Abstrakt – Abstract

The literature review of this thesis was focused on the pathway of folate biosynthesis with enzymes involved in it and factors effecting the synthesis of folate by bacteria. The literature was also partly focused on the introduction to propionic acid bacteria (PAB) and the folate production by these bacteria.

The aim of the experimental part of the thesis was to screen the folate productivity of selected PAB strains isolated from various dairy and cereal sources after 96 hours anaerobic fermentation and also to see the effect of precursor (*para*-aminobenzoic acid) of folate biosynthesis on folate production by the strains. Then to further investigate the folate production of some promising PAB strains from screening part either with glucose or lactate as carbon source at four growth phases in aerobic fermentation. The propionic agar medium was used with either glucose or lactate as carbon source in the medium. Optical densities, pH, cell masses were measured after fermentation and folate produced by the strains was determined from biomass and supernatant of the samples using microbiological assay. Carbon consumption and metabolic end-products were analysed with HPLC after fermentation.

Some of the screened PAB strains were promising folate producers. Strain 257 produced folate up to 124 µg/l which is even higher than production by some good Lactic acid bacteria (LAB). PAB strains produced intracellular folate upto 28954 ng/g cell biomass and excreted folate into medium upto 107 ng/ml. Strains grew faster with lactate than glucose but cell masses were higher with glucose than lactate even in the low pH. PAB strains showed the highest folate productivity in anaerobic fermentation with lactate as carbon source and aerobic fermentation with lactate as source was observed to be the best for high organic acid production. However, further studies are needed to optimise the cultivation condition of selected PAB strains for their best folate production in different matrices.

Avainsanat – Nyckelord – Keywords
Folate, synthesis, fermentation, *para*-aminobenzoic acid, carbon source, propionic acid bacteria

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PREFACE

This Master's thesis was carried out at the Department of Food and Environmental Sciences of University of Helsinki. The study was a part of a project called 'Natural fortification of foods: Microbial in situ synthesis of vitamin B12 and folate in cereal matrix' which is funded by the Academy of Finland.

First of all, I would like to thank Professor Vieno Piironen who gave me the opportunity and the topic to carry out my master thesis. I would like to thank Professor Kati Katina to provide me the platform and guidance to start my thesis. I have my heart full of gratitude and acknowledgement to my supervisors Ph. D. Minnamari Edelmann and M. Sc. Paulina Deptula for all the valuable times they have given me to instruct and guide me through all the learning procedures and difficulties in experimental laboratory. Only with their proper guidance and patience, I was able to complete my practical works. I would again like to express my gratitude to my supervisor Ph. D. Minnamari Edelmann for her continuous effort to improve my thesis writing. I need to thank my friend M. Sc. Mamata Bhattarai for helpful discussions about statistical analysis. I am thankful to all of my colleagues during laboratory work and friendly environment.

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Subash Basnet

List of abbreviations

CO_2	Carbon dioxide
DHPPP	Dihydroneoptrin triphosphate
DHP	Dihydropteroate
DHF	Dihydrofolate
FOS	Fructo-oligosaccharides
GOS	Galacto-oligosaccharides
GTP	Guanosine triphosphate
HPLC	High pressure liquid chromatography
KEGG	Kyoto Encyclopedia of Genes and Genomes
LAB	Lactic acid bacteria
LC-MS	Liquid chromatography- mass spectrometry
MA	Microbiological assay
MBA	Microbiological method
OD	Optical density
PAB	Propionic acid bacteria
*p*ABA	*para*- amino benzoic acid
PGA	Pteroylmonoglutamic acid
PPA	Propionic growth medium
PBS	Phosphate buffer solution
RDI	Recommended daily intake
RI	Refractive Index
THF	Tetrahydrofolate
UV	Ultraviolet

1 INTRODUCTION

Folate is a water-soluble B- vitamin which has an essential nutritional function in the human body. Folate is the collective name of a group of substances with a chemical structure related to pteroylmonoglutamic acid (PGA or folic acid). Folic acid is stable, chemically synthesized compound that is used in vitamin preparations and it is added to foods. Folate is involved in many metabolic pathways, mainly in carbon transfer reactions such as purine and pyrimidine biosynthesis and amino acid conversions. It has essential functions in cell metabolism such as DNA methylation and repair, synthesis of nucleotides, vitamins and amino acids (LeBlanc et al. 2007; Iyer and Tomar 2009). Because of this vital role, large quantity of folate is needed during the process of rapid cell division such as during development of foetus.

Folate deficiency is an important issue in many parts of the world, particularly where there is poverty and malnutrition. It plays key role to cause anaemia after nutritional iron deficiency. A low folate intake has been associated with a number of health disorders such as Alzheimer's and coronary heart diseases, osteoporosis, increased risk of breast and colorectal cancer, poor cognitive performance (Mason 1995; Morrison et al. 1995; Boushey et al. 1996; Ames 1999; LeBlanc et al. 2007). Folate deficiency has been known to be the main cause of neural tube defects during the embryo development (Daly et al. 1995). The recommended daily intake (RDI) as approved by European Union is 400 µg/d for adults. Even higher intake (600 µg/d) has been recommended for women during pregnancy and 500 µg/d for lactating women (FAO/WHO 2002; IOM 2004).

Green leafy vegetables, liver, legumes, egg yolk, wheat germ, milk and milk products and yeast are the major sources of folate (Eitenmiller and Landen 1999; Lin and Young 2000; Witthöft et al. 1999). The Food and Drug Administration of the U.S. Department of Health and Human Services has implemented mandatory regulation that cereal-grain products should be fortified with folic acid at 140 µg/100g of cereal grain products to reduce the incidence of neural tube defects pregnancies in United States (Tamura 1998). More than 60 countries around the world practice mandatory folate fortification programs to ensure the prevention of diseases and disorders related to folate deficiency. However, mandatory fortification is not in-forced in the European Union. On the other hand, there has been a debate and doubt that high intake of folic acid could delay the diagnosis of vitamin B12 deficiency (Bailey et al. 2003; Jägerstad 2012).

Plants, fungi and microbes are able to synthesize folate. However, humans and other animals are incapable of synthesizing folate making it an essential nutrient that has to be consumed from food sources or as supplements (Donnelly 2001). Natural folate enhancement in cereals and dairy products by introducing folate producing microorganism has gain more attention in these recent days. Lactic acid bacteria (LAB) and yeasts are good folate producers among food grade

microorganisms producing upto 100 µg/l and 145 µg/g dm cell mass respectively. (Hugenholtz et al. 2002; Sybesma et al. 2003b; Hjortmo et al. 2005; Iyer and Tomar 2009). Total folate production upto 256 µg /l by genetically engineered *L. lactis ssp. lactis* was reported by Sybesma et al. (2003a). There are also few studies on folate production by propionic acid bacteria (PAB). PAB strains have been found to produce folate during milk fermentation with equal or even higher production than some dominating folate producing strains (Hugenholtz et al. 2002; Hugenschmidt et al. 2011). In addition, PAB are well known as efficient vitamin B12 producers and they are often used for industrial vitamin B12 production (Hugenschmidt et al. 2011). Since the metabolisms of folate and vitamin B12 are interdependent, studies have been conducted to produce higher amount of both vitamins in co-culturing of LAB and PAB (Hugenschimdt et al. 2011). Genetic engineering of some natural strains has found to increase total folate levels but ethical issues and governmental regulations are not yet ready to issue permission on implementing the ideas on market. Hence natural selection of higher folate producers and use of them in-applications could be in the high demand in the food industry (Edelmann 2014).

In this thesis, the aim of literature review was to give an overview about the folate structure, chemistry and bioavailability and further to become familiar with folate biosynthesis and factors effecting the synthesis of folate by bacteria. In addition, the objective of the literature review was to provide general introduction to PAB and folate production by those bacteria. The aim of the experimental research in this thesis was to examine folate production of selected PAB isolated from various dairy and cereal sources after 96– hour anaerobic fermentation and also to see the effect of precursor (*para*-aminobenzoic acid) of folate biosynthesis on folate production by the strains. Then, further investigate the folate production with selected promising PAB either with glucose or lactate as carbon source at four growth phases in aerobic fermentation.

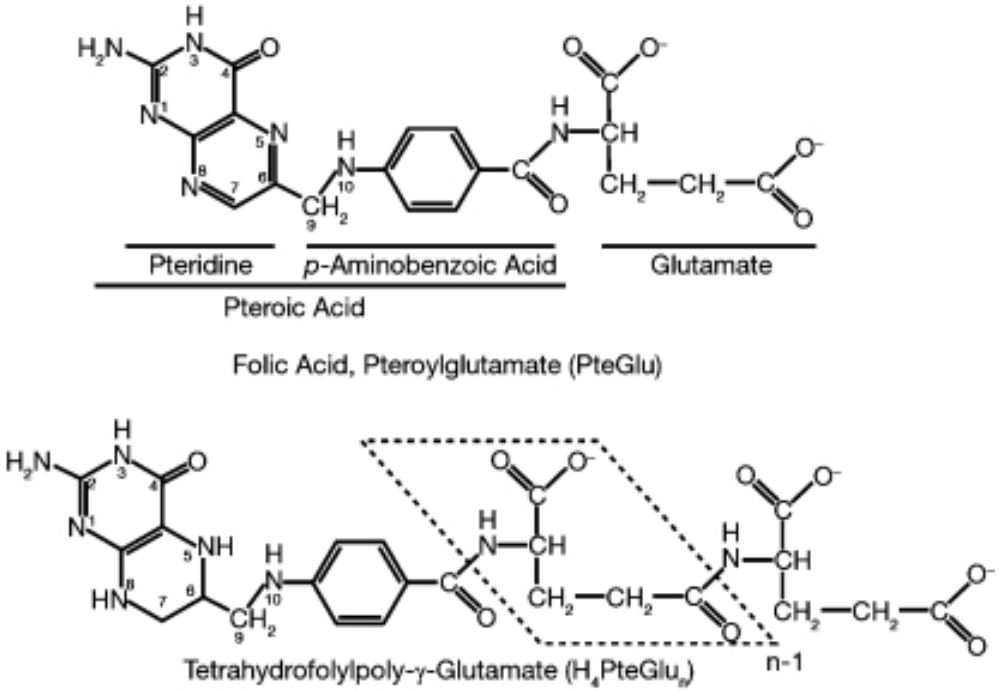
2 LITERATURE REVIEW

2.1 Folate

2.1.1 Structure and chemistry

Folate is a generic term for all the derivatives which have similar biological activity to folic acid, including naturally present folate polyglutamates in foods. Folic acid is a synthetic form which is commonly used for food fortification and nutritional supplements. Folic acid (PGA) consists of pteridine ring (2-amino-4-hydroxy-methylpterin) joined to *para*-aminobenzoic acid (*p*ABA) through methylene bridge. One or more L-glutamate residues are conjugated to *p*ABA

with peptide linkage. (Fig. 1). The forms of folates occurring in the nature differ in the extent of the reduction state of the pteroyl group, substituents on the pteridine ring and the number of glutamate tails attached to the pteroyl group (LeBlanc JG 2007; Edelmann 2014). The naturally occurring folates include 5-methyltetrahydrofolate (5-MTHF), 5-formyltetrahydrofolate(5-formyl-THF), 10-formyltetrahydrofolate(10-formyl-THF), 5,10-methylenetetrahydrofolate (5,10-methylene-THF), 5,10-methenyltetrahydrofolate (5,10-methenyl-THF), 5-formiminotetrahydrofolate (5-formimino-THF), 5,6,78-tetrahydrofolate (THF) and dihydrofolate (LeBlanc 2007). Over 100 distinct forms of this vitamin may exist naturally as a result of this chemical diversity, although fewer than 50 principal forms of folate exist in most animal and plant tissues (Gregory 1989). Most naturally occurring folates are pteroylpolyglutamates, containing two to seven glutamates joined in peptide linkages to γ-carboxyl of glutamate. The principal intracellular folates are pteroylpentaglutamates, while the principal extracellular folates are pteroylmonoglutamates. Pteroylpolyglutamates with up to 11 glutamic acid residues exist naturally (LeBlanc 2007).



One Carbon Substituent		Position	Oxidation State
Methyl	—CH_3	N-5	Methanol
Methylene	—CH_2—	N-5, N-10	Formaldehyde
Methenyl	—CH=	N-5, N-10	Formate
Formyl	—CHO	N-5 or N-10	Formate
Formimino	HN=CH—	N-5	Formate

Fig 1. Structure of folic acid (pteroyl-L-glutamic acid) and native food folates as polyglutamyl tetrahydrofolates with different one-carbon substituents.

There are considerable differences in stability between various reduced folates. Folic acid shows substantially greater stability than the reduced folates. The order of stability of these reduced folates are 5-formyl-THF > 5-methyl-THF > 10-formyl-THF > THF (Forssén 2000). The chemical reactivity of some important folate compounds makes the vitamin one of the most sensitive to losses during food processing. Considerable losses have been reported following a number of different processes (Gregory 1996).

2.1.2 Bioavailability

Bioavailable folate is a proportion of the ingested folate that is available for utilisation in normal physiological functions, and for storage (Öhrvik and Witthöft 2011; Gregory 2012). Folates as their monoglutamates are absorbed by an active energy-dependant, carrier-mediated process at physiological concentrations and by passive absorption at higher concentrations (Selhub and Rosenberg 1981). Absorption takes place mainly in the jejunum and is markedly influenced by pH with maximum absorption at pH 6.3. The polyglutamic folates must be cleaved to their monoglutamate forms by a pteroylpolyglutamate hydrolase, referred to as folate deconjugase, before uptake can take place in the intestinal epithelial cells. Reisenauer et al. (1977) reported the existence of two separate folate deconjugase activities in human jejunal mucosa: one soluble and intracellular, the other membrane-bound and concentrated in the brush border. This enzyme is present in sufficient amount and does not limit the folate absorption. Folate can be absorbed and transported as a monoglutamate into portal vein. The polyglutamate forms have bioavailability up to 60 - 80% compared with the monoglutamate forms (Gregory 1995) and thus absorption efficiency of natural folates is approximately half from that of synthetic folic acids (Sauberlich et al. 1987). Folate binding proteins from milk may act to increase efficiency of folate absorption by protecting dietary folates from being used by bacteria, thus increasing absorption of folates (Eitenmiller and Landen 1999). Other dietary interaction which could possibly effect on folate absorption include effect of foods on intestinal pH potentially modifying conjugase activity, presence of folate antagonists, intestinal changes influenced by alcohol or chelation. (Selhub and Rosenberg 1997; Gregory 1997; McNulty and Pentieva 2004).

2.2 Determination of folate

The analysis of folate has an increasing importance due to its significant role in health and diseases. Due to its multiple forms, low stability and presence in lower concentration in biological systems, its analysis is a challenging task. Generally, the assay of folates involves three steps: liberation of folates from cellular matrix; deconjugation from polyglutamate to the mono and di-glutamate forms; and the detection of the biological activity or chemical concentration of the resulting folates (Arcot and Shrestha 2005) which requires three methodological process: extraction; enzyme treatment; and quantification.

Extraction

Generally, sample preparation involves grinding of sample and homogenisation with a suitable buffer system followed by heating and centrifugation. Heating during the extraction procedure causes thermal denaturation of folate binding proteins and enzymes that may catalyse the folate degradation or interconversion (Keagy 1985; Gregory 1989). Several kinds of heat treatments have been used such as boiling water bath, autoclaving and microwave heating. The pH of the buffer has been 4.5 - 7.5 depending upon the optima of the enzymes used during the later deconjugation step (Keagy 1985; Gregory 1989). Heating may destroy the folate derivatives but the degree of the destruction is mainly influenced by the pH of the buffer, reducing agents in the buffer and type of buffer. In most of the folate assays, a preservative is added to the extraction buffer to prevent the oxidative loss of labile reduced folates. Ascorbic acid and 2-mercaptoethanol are two most common anti-oxidants used in the folate extraction buffer. (Arcot and Shrestha 2005). It has been observed that the stability of reduced folates during extraction increases when ascorbic acid and 2-mercaptoethanol are added together (Vahteristo et al. 1996).

Enzyme treatment

Most of the natural folates occur in the polyglutamate forms but normally they are determined as their monoglutamates. In microbiological assay (MA), a test organism, *Lactobacillus rhamnosus* responds equally through mono- to tri-glutamates but much more slowly to longer-chain derivatives (Tamura et al. 1972). High pressure liquid chromatography (HPLC) system is a popular technique for separation and quantification of individual folates. Native reduced folate forms have been separated and quantified by HPLC by several researchers (Vahteristo et al. 1997; Kariluoto et al. 2001). Conversion of polyglutamates to mono- or diglutamates requires γ-glutamylcarboxypeptidase (conjugase or folate hydrolase). Once they are

hydrolysed, these folates support growth of *L.rhamnosus* and are also ready to be quantified by other methods (Arcot and Shrestha 2005). Conjugases isolated from chicken pancrease or hog kidney are the most commonly used sources of the conjugases. Conjugase from human and rat plasma, rat pancreas and rat liver have been used to a lesser extent (Keagy 1985). The activity of these conjugases differs in their pH optima, mode of action and specificity for folate derivatives (Krisch and Chen 1984; Gregory 1989).

Treatment with only folate conjugase is not enough to liberate folates trapped in or bound to the matrices of proteins and polysaccharides. Therefore proteolytic and amylolytic enzymes are used to increase the liberation of folates. De Souza and Eitenmiller (1990) and Martin et al. (1990) were the first ones who reported a method where, in addition to the traditional treatment with folate conjugase, protease (EC 3.4.24.31) and α-amylase (EC 3.2.1.1) were also used. The extraction method was named as the 'tri-enzyme treatment'.

Quantification

Microbiological assay (MA)

The fact that certain microorganisms require nutritional factors that they are unable to synthesize themselves, led to the applications where these microorganisms are used for quantitative determination of vitamins in the early 1940s (Arcot and Shrestha 2005). Although there have been several alternative methods for determining folates in foods and biological samples, the MA is still popular method for folate analysis. Several improvements have been introduced since its initial introduction to overcome its drawbacks for maximal retention of folate in samples. The use of 96-well microtiter plates and cryoprotection of test organism in glycerol has improved the efficiency and reproducibility and lowered the detection limit. The presence of antibiotics or antifolates can interfere with measurement. This problem, however, has mainly concerned blood samples (Edelmann 2014).

MA is based on the growth of the microorganisms which requires folate as a nutrient thereby growth could be measured turbidimetrically. *L.rhamnosus* is the most common assay organism for folate analysis because it responds to the widest variety of folate derivatives (Bird and McGlohon 1972; Krumdieck et al. 1983). It can fully utilise mono-, di- and triglutamates, but after triglutamate, its response decreases significantly with increasing length of glutamyl tail (Pfeiffer et al. 2010). Although L. rhamnosus has generally been reported to exhibit a similar growth response to various monoglutamates, few studies suggested that its response differs to various folate derivatives. Weber et al. (2011) reported that the response of *L. rhamnosus* to 5-HCO-H_4folate was the highest followed by 10- HCO-H_4folate, PGA, 5-CH_3-H_4folate and

H_4folate. One of the major drawback with *L.rhamnosus* method is the possibility of microbiological contamination. Proper aseptic working condition should be used.

Liquid chromatography (LC)

Liquid chromatography methods are used for the separation of different folate vitamers. These techniques involve two steps: separation and purification of deconjugated extract, and detection and quantification of eluted monoglutamates (Gregory 1989). Sensitive detection techniques are given more emphasis due to the low concentrations of folate present in most foods or other tissues. Folate vitamers have been generally detected by ultra violet/diode array detectors (DADs) and fluorescence detectors (FLR) (Edelmann 2014). High pressure liquid chromatography (HPLC) methods separate folate vitamers as their monoglutamates based on reverse-phase chromatography. Generally, octadecyl (C18) bonded silica has been used as stationary phase material (Pfeiffer et al. 1997; Freisleben et al. 2003; Kariluoto et al 2001, 2004; Gujska and Kuncewicz 2005; Gujska and Majewska 2005). Retention of monoglutamates on these columns decreases rapidly above pH 4 and 5 (Lucock et al. 1995). In most of the LC methods, the mobile phase therefore consists of a phosphate buffer at pH of around 2-3 and acetonitrile (Pfeiffer et al. 2010).

Ultrahigh pressure liquid chromatography (UHPLC) utilises columns packed with small-diameter particles (1.8 μm) creating high pressure during analysis. UHPLC gives a significant improvement in the resolution per time unit and faster analysis compared to HPLC methods. Jastrebova et al. (2011) reported that the run time was 4 times shorter and limit of detection values were lower with UHPLC than with HPLC.

Liquid chromatography- mass spectrometry (LC- MS)

HPLC techniques with ultraviolet and/or fluorescent detection determine detect the different forms of folate. According to previous literature, total folate content obtained by HPLC has been lower (30-50%) than that determined by MA (Kariluoto et al. 2001; Ginting and Arcot 2004). Improved method for analysing different forms of folates is required because these compounds differ in their bioavailability (Tamura and stokstad 1973; Finglas et al. 2006). Recently, LC combined with mass spectrometry (MS) applications have also gained increasing attention for the analysis of folate vitamers (Edelmann 2014). LC-MS analysis is considered most accurate when internal standards are used to correct for losses of the vitamers during sample clean-up (Vishnumohan et al. 2011). The precision and accuracy of LC-MS folic acid

in quantification in cereal matrices has compared favourably with values obtained through MA (Pawlosky et al. 2003). LC-MS methods are not limited by the low fluorescence activity of PGA and 5-HCO-H_4folate (Edelmann 2014). Further development and validation of LC-MS for specified samples is being taken into attention by researchers because there is still the possibility of matrix effect which must be overcome for different samples (Arcot and Shrestha 2005).

2.3 Folate biosynthesis

2.3.1 Enzymes and genes of synthesis

Plants and microorganisms have the ability for *de novo* synthesis of folates, making them the main dietary source of folate for humans and animals (Blancquaert et al. 2010). Animals cannot synthesize folates and assimilate these derivatives with a diet exploiting active transport systems (Rossi et al 2011). The folates biosynthesis pathways in plants and microorganisms are similar, except that in plants the process is split among three subcellular compartments (chloroplast, cytosol and mitochondria). In plants, the pteridine moiety is synthesized in the plant cytosol, *p*ABA is synthesized in the chloroplast and folates are synthesized from these two precursors in mitochondria. In addition to biosynthetic enzymes, membrane-bound transporters for folates and various pathway intermediates must be present. (DellaPenna 2007; Edelmann 2014).

For *de novo* biosynthesis of folate, both the precursors' dihydroneoptrin triphosphate (DHPPP) and *p*ABA are essential whereas the third component glutamate is normally taken from the medium. In folate biosynthesis, two condensation reactions take place to form ultimate THF. One is the condensation of *p*ABA with DHPPP to produce dihydropteroate (DHP) and secondly, the reaction of glutamate with DHP to form dihydrofolate (Wegkamp et al. 2007; Iyer and Tomar 2009).

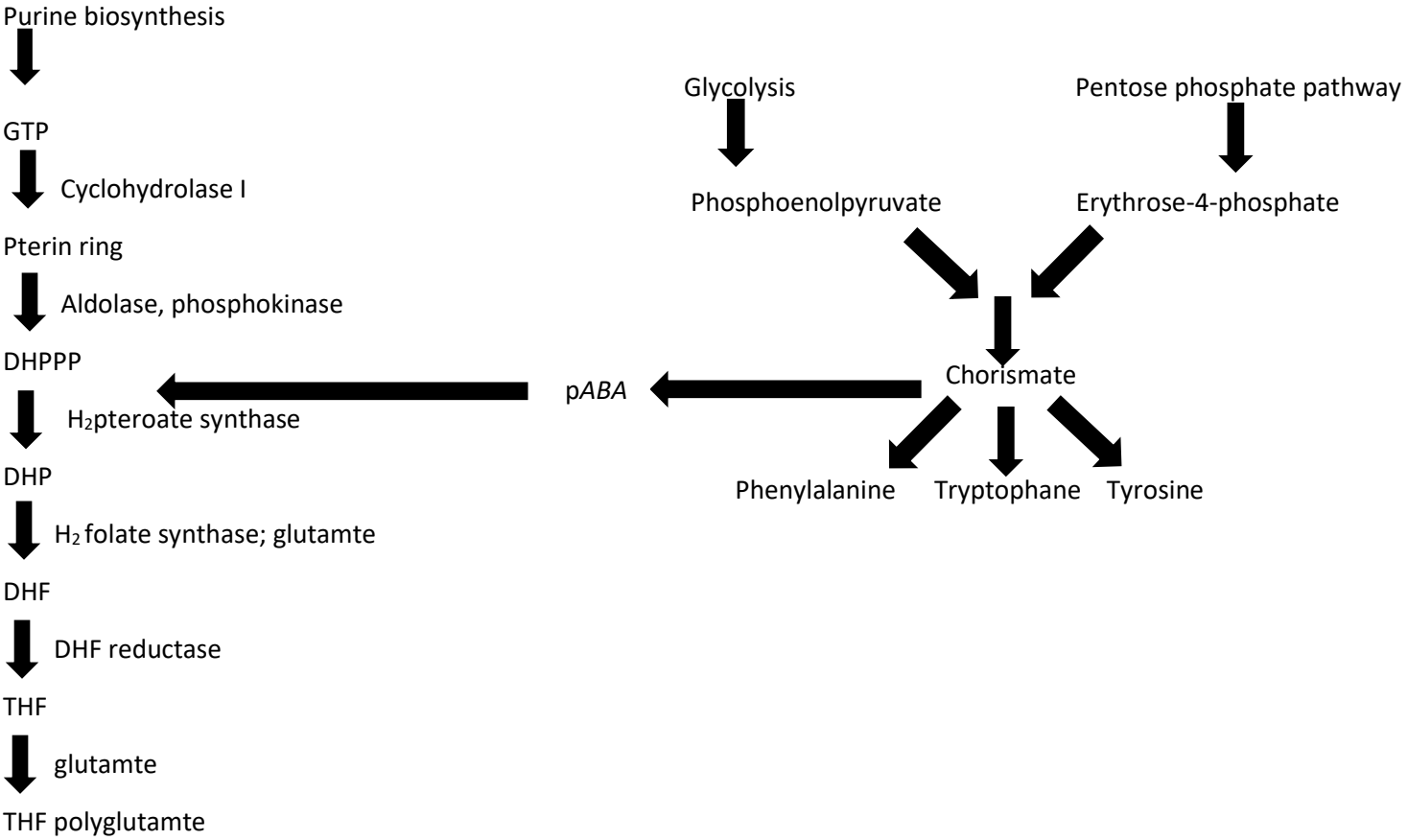


Figure 1. Biosynthesis of folate including pathway of *p*ABA and incorporation of glutamate tail to form tetrahydrofolate (Wegkamp et al. 2007; Sybesma et al. 2003).

The *de novo* synthesis of folate requires several consecutive reactions to form a biologically active cofactor THF. The pteridine synthesis starts with the conversion of guanosine triphosphate (GTP) into DHPPP in purine biosynthesis pathway. *p*ABA is synthesized from chorismate in shikimate pathway. Erythrose 4-phosphate and phosphoenolpyruvate react to form shikimate-3-phosphate which transformed to chorismate. Chorismate serves as a branching point for synthesis of the aromatic amino acids (tryptophan, phenylalanine, tyrosine) and *p*ABA (Wegkamp et al. 2007; Rossi et al. 2011).

The process of *de novo* synthesis of folate is mediated and induced by group of enzymes at various steps. Conversion of GTP to pteridine induced by GTP cyclohydrolase I which is followed by aldolase and pyrophosphokinase reactions to give activated DHPPP. Then the first condensation reaction occurs between DHPPP and *p*ABA mediated by dihydropteroate synthase and DHP is formed. DHP reacts with glutamate assisted by dihydrofolate synthase to yield dihydrofolate (DHF). Then DHF reductase reduces DHF to a biologically active cofactor THF. Addition of multiple glutamate tails by folypolyglutamate synthase leads to THF polyglutamates (Wegkamp et al. 2007; Rossi et al. 2011).

Rossi et al. (2011) have established in their review that the microbes which possess all the genes encoding enzymes for the biosynthesis of DHPPP and chorismate are capable to folate production. By using KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways maps database, ability of microbes to synthesize *p*ABA *de novo* could be predicted (www.genome.jp/kegg). Based on the the sequenced genes, the genera *Lactobacillus* show inability to synthesize *de novo p*ABA. In addition only strains of *L. plantarum* show complete shikimate pathway for chorismate production while it is absent or partially absent in all the other lactobacilli (Rossi et al. 2011).

LAB species *Lactococcus lactis*, *Streptococcus thermophiles* and bifidobacteria species *Bifidobacterium. adolescentis* and *Bifidobacterium. dentium* possess all the genes for both the shikimate pathway and chorismate conversion into *p*ABA which make them able to produce folate even when there is no *p*ABA available in the cultivation medium. Species like *L.plantarum* and *L. longum* which are lacking enzymes necessary for chorismate conversion into *p*ABA could produce folate when they are cultivated with supplemented *p*ABA in the medium while other species which lack both genes for DHPPP synthesis and the gene encoding DHPPP transformation into DHP are expected to be auxotrophic for folates and remain incapable of folate production even in the presence of *p*ABA (Lin and Young 2000; Hugenholtz and Smid 2002; Rossi et al. 2011).

Regarding PAB strains, KEGG pathway database (www.genome.jp/kegg) shows that the most of the species (*Propionibacterium acidipropionici, Propionibacterium freudenreichii* subsp. *Shermanii* CIRM- BIA1*, Propionibacterium freudenreichii* subsp. *Freudenreichii* DSM 20271) from genus propionibacterium possess complete or partial enzymes responsible for DHPPP synthesis and chorismate conversion into *p*ABA making them possible and promising folate producers. However, even if species harbours all the genes required for folate biosynthesis, there may be differences among strains in terms of folate production. Thus level of folate production, accumulation of folate inside cell, excretion of folate into the medium, distribution of vitamers depend on the individual strain (Lin and Young 2000; Crittenden et al 2003; Sybesma et al. 2003b; Rossi et al. 2011).

According to Sybesma et al. (2003a), only five or six genes in the folate gene cluster favoured in folate biosynthesis in results of sequencing and annotation of folate gene cluster. They are: *fol* A (encode dihydrofolate reductase), *fol* B (predicted to encode neopterine aldolase), *fol* K (predicted to encode 2-amino-4 hydroxy-6-pyrophosphokinase), *fol* E (predicted to encode cyclohydrolase I), *fol* P (predicted to encode dihydropteroate synthase), *fol* C (predicted to encode folate synthase). *L. lactis* is the only species for which all folate biosynthesis genes are studied till date (Iyer and Tomar 2009).

2.3.2 Cultivation factors affecting folate production

With the advancement in the computational biology, science has been able to predict results of various biological systems with previously collected data. Similar process is applied in regards of knowing folate biosynthesis possibility in microorganisms by determining their genome sequence. The microbes with the availability of all the genes required for folate biosynthesis would be the most likely to be selected for folate production.

Even though microbes harbour all the responsible genes for folate biosynthesis, it is not a guarantee for folate production. Different cultivation condition and various physical and nutritional factors such as the composition of the medium, co-culturing, temperature, pH, incubation time and presence of folate precursor have been reported to affect folate production.

*p*ABA and glutamate

*p*ABA and glutamate are among the important precursor to make folate biosynthesis possible in plants and microorganisms. Many LAB and PAB strains have been found to lack ability to synthesize *p*ABA due to complete or partial absence of enzymes necessary for chorismate conversion into *p*ABA (KEGG database). There are a few studies that have been carried out mainly to know the effect of *p*ABA on folate production by LABs and PABs (Sybesma et al. 2003; Pompie et al. 2007; Santos et al. 2008; Nor et al. 2010; Gangadharan and Nampoothiri 2011; Hugenschmidt et al. 2011).

L. lactis has been shown its dependency on concentration of *p*ABA in a few previous studies. Addition of *p*ABA (14 mg/L) in chemically defined medium increased the folate production by two-fold (Sybesma et al, 2003). When Bifidobacteria were cultured with different *p*ABA concentrations (0.3 mM-100mM), the highest biomass yield and the highest folate production was obtained at 0.3 mM *p*ABA, whereas growth was inhibited and folate production was decreased with increased concentration to 100 mM (Pompei et al. 2007). In the study by Hugenschmidt et al. (2011), addition of *p*ABA (10 mg/l) had accountable stimulating effect on folate production without affecting cell growth. The folate yield was upto 10-20 fold higher than without *p*ABA addition. *p*ABA exhibited the best folate increment when it was added in food matrices with concentration of 10 mg/ml (Gangadharan and Nampoothiri 2011) but as mentioned by Pompie et al. (2007), *p*ABA inhibited the folate biosynthesis when it was added in higher concentration. Addition of glutamate (10 mg/ml) increased folate production by *L. Lactis* subsp. *cremoris* in skim milk (Gangadharan and Nampoothiri 2011) while it decreased the folate production when added with combination of *p*ABA (hugenschmidt et al. 2011).

Co-culture production

The use of combination of different strains of bacteria may be more efficient than the use of mono-culture. Crittenden et al. (2003) reported a six fold increase in folate production in a skim milk medium when a mixed culture (*B. animalis* CSCC 1941 and *Streptococcus thermophiles* CSCC 2000) was used. Similarly, there was 1.5-fold increase in the extracellular folate when co-culture of *L. plantarum* and *P. freudenreichii* was used compared to monoculture of PAB or LAB strain (Hugenschmidt et al. 2011). Likely, the mixture of *L. delbruecki* subsp. *bulgaricus* strain and *S. thermophiles* strains in milk (Laino et al. 2013) and combination of strains of *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* gave the best results in Domiati cheese (Ayad 2009). Even combination of LAB strains with yeasts have been reported to increase the folate production (in 3.5% oat bran solution with 2% added glucose) six fold more than LAB or yeast alone (Korhola et al. 2014). Similarly, Kariluoto et al. (2006) reported the highest folate concentrations in sourdoughs made from rye flour with non-sterile control containing naturally occurring bacteria and yeast rather than with LAB monocultures.

Time, temperature and pH

There are various physical factors which affect the quantity of folate production by microorganisms. The effect of intracellular and extracellular pH on folate production by microbes has been reported in a few studies. Folate biosynthesis was more likely limited by low pH leading to cessation of growth and even death of cells at pH values below 4 and 5 for LAB and PAB, respectively, (Hettinga and Reinbold 1972; Hammes and Hertel 2009). Under controlled pH 5.5, excretion of folate into the medium was favoured by *S. thermophiles* but not by *L. lactis.* Folate production of *L. lactis* and *S. thermophiles* increased up to three-fold with increasing pH from 5.5 to 7.5 under controlled growth conditions (Sybesma et al.2003b). It has been suggested that higher pH delays acidification of medium prolonging growth of microbes and maintains intracellular pH for more efficient folate biosynthesis (Sybesma et al.2003b). On the other hand, Pompie et al. (2007) did not observe any effect of pH on folate production by *B. adolescentis* in the pH range from 5.7 to 6.9. Hugenschmidt et al. (2010) also reported that the effect of pH on folate biosynthesis depends on strains. *P. jensenii* and *P. thoenii* produced lower concentrations of folate below pH 5.3 while *P. acidipropionici* yielded 7 times more folate despite of low pH value of 5.0 after 72 hour of incubation. *P.freudenreichii* and *Propionibacterium sp*. had pH value between 5.5 and 6.2 after 72 hour fermentation and folate yield was moderate.

In a few studies, different cultivation temperatures for folate biosynthesis have been tested. Fermentation at 37 °C led to higher folate production than fermentation at 30 °C (Holasova et al. 2005) or at 30 °C and 42 °C (Gangadharan and Nampoothiri 2011). However, Tomar et al. (2009) showed that folate production of *S. thermophiles* cultivated in skim milk was higher at 42 °C than at 30 °C or at 37 °C. According to Kariluoto et al. (2010), *P. agglomerans* ON3 produced more folate at 28 °C at pH 5.5 than at pH 7 while folate content was higher at pH 7 than at pH 5.5 when temperature was 37 °C.

Long fermentation has been observed to decrease the folate levels in yeasts. Intracellular folate content decreased after 20 hour fermentation until stationary phase was reached (Hjortmo et al. 2008a). Holasova et al. (2004) as well as Lin and Young (2000) reported maximum folate production for LAB during the first 6-12 hour cultivation. After that folate levels decreased up to 18 hour, probably due to folate consumption by the cells. By contrast extracellular folate concentration increased for LAB and PAB during longer incubation time (Hugenschmidt et al. 2010). The increase in folate content in the medium, might be due to autolysis of the cells (Kariluoto et al. 2010). Kariluoto et al. (2010) reported that folate concentration at stationary phase were lower than those at the exponential phase for *Pantoea ananatis* ON1, *Bacillus subtilis* ON5, and Propionibacterium sp. RB9.

Composition of medium

The composition of cultivation medium may limit and stimulate folate biosynthesis. Therefore, folate production can be increased by selection of optimal composition of cultivation medium.

Researchers are keen to know the best carbon source for microbes in their folate biosynthesis. The capability to ferment different carbon sources is species-dependent and strain-dependent. To be able to catabolise certain carbon source, bacterial strains require a functional transport system (Falentin et al. 2010). PAB can grow by obtaining energy from various carbon sources but biomass yield depend upon carbon nutrient (Zárate and Chaia 2012). Nor et al. (2010) reported that lactose was a better carbon source to *L. plantarum* strain in a modified MRS medium than glucose and maltose. However, the best lactose concentration for folate production appears to depend on the strain (Tomar et al. 2009; Iyer et al. 2010). Lactate is definitely the main energy source for Propionibacterium strains (Piveteau 1999).

Lactose addition at a concentration of 1% led to 17% increase in folate production by *S. thermophiles* when it was incubated at 42 °C. By contrast, higher dose of lactose decreased the folate production (Tomar et al. 2009). Lin and Young (2000) observed that selected LAB strains and Bifidobacteria *(L. acidophilus, L. bulgaricus, S. thermophiles and B. longum*) had

2-2.5 fold increase in folate level in reconstituted non-fat dry milk than in a lactose-free, complex medium. The addition of prebiotics such as sorbitol and mannitol separately into the skim-milk medium increased slightly folate production by *L. lactis* sbsp. *cremoris* (Gangadharan et al. 2011) while addition of fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), glucose, fructose, raffinose and lactose did not lead to differences in folate production by some selected LAB strains and Bifidobacteria (Pompie et al. 2007; Padalino et al. 2012).

Folate production may be stimulated by cultivating the cells under growth limiting conditions. Sybesma et al. (2003b) reported that the addition of NaCl decreased the growth rate and increased the folate production in both *L. lactis* and *S. thermophiles*. The reason for increased folate production when growth was inhibited is not clearly known. Probably, guanosine triphosphate (GTP), one of the folate precursors, accumulates due to decrease in DNA and RNA synthesis (Sybesma et al. 2003b).

Pompei et al. (2006) studied the effect of exogenous folate concentration on folate production and found that higher folate concentration in medium effected negatively on folate productivity in some strains. Results were also strain dependent. Bifidobacteria were cultivated in the presence of folate in a range between 0 and 50 ng/ml. High exogenous folate concentration showed negative effect on folate production by *B. adolescentis* MB 114 and MB 115 and *B. pseudocatenulatum* MB 116 whereas negative effect was not observed for *B. adolescentis* MB 227 and MB 239 and *B. pseudocatenulatum* MB 237.

Metabolic engineering of microbes

To design a rational approach for metabolic engineering of folate production, proper understanding of the pathways, enzymes and genes involved in folate biosynthesis is required (Sybesma et al. 2003a). *Lactococcus lactis* is by far the most studied LAB, and over last decades a number of refined and efficient methods have been developed for this starter bacterium. These methods have crucial importance in metabolic engineering strategies that aim at inactivation of undesired genes or overexpression of existing or novel ones (Swindell et al. 1996; Sybesma et al 2003a). For an example, Sybesma et al. (2003) reported threefold increase in the total folate production by *L. Lactis* when over-expressing the folate gene cluster, which encodes GTP cyclohydrolase I. Folate production in *L. lactis* increased up to 80-fold as a result of combined over-expression of the *p*ABA gene cluster and the folate biosynthesis genes (Wegkamp et al. 2007).

2.4 Propionic acid bacteria and folate production

2.4.1 Introduction to propionic acid bacteria

PAB are gram positive, non-spore forming, non-motile rod shaped, generally catalase positive bacteria. They are anaerobic to aero-tolerant (Poonam et al. 2012). PAB strains are originally classified in the genus *Propionibacterium* and it is divided into dairy propionibacteria (isolated from dairy products) and cutaneous propionibacteria (typically found on skin) based on their habitat. Out of 12 species of *Propionibacterium*, 4 strains (*P. freudenreichii, P.acidipropionici, P.thoenii, P.jensenii*) are typical dairy species (Cummins and Johnson 1986). Dairy propionibacteria have been regarded as GRAS (Generally recognized as safe) and QPS (Qualified presumption of safety) since long history (Meile et al. 2008).

Dairy propionibacteria are used for several industrial purposes due to their ability to convert lactate and carbohydrates to propionic acid, acetic acid and carbon dioxide (CO_2) (Playne 1985). PAB are primarily used as starter culture for the manufacture of Swiss type cheeses where they are responsible for the eye formation and characteristic flavour during ripening process (Rehn et al. 2011). Eye formation is the result of CO_2 production diffusing the surface matrix of cheese and flavour production is characterized by the metabolism of lactate, propionate, acetate and CO_2 (Langler et al. 1967; Thierry and Maillard 2002).

Antimicrobial compounds produced by food grade bacteria are an effective method to preserve food in natural way. PAB produce bacteriocins and organic acids which are widely used for food preservation. Propionicin and jenesniin are the major bacteriocins produced by propionibacteria which are named after their species name (Holo et al. 2002). Propionic acid and its salts which are of the major metabolic products of the microbial fermentation by propionibacteria are very important in food preservation and manufacture of various products like cellulose, perfumes, herbicides, pharmaceuticals, fibers etc. Since commercial propionic acid production via petrochemical method by hydrocarboxylation of ethylene is not environment friendly and is costly, microbial production of propionic acid using low cost materials has got high interest to meet consumers demand (Liu et al. 2012). Propionic acid is produced via dicarboxylic acid pathway with acetic acid and succinic acid as the main by-products (Poonam et al. 2012).

PAB have long been considered safe and studied for health benefits after their consumption. Microbes having ability to survive Gastrointestinal (GI) transit, adhesion to epithelial mucosa and provide health benefits to the host are called probiotic organism (Huang et al. 2003; Uchida et al. 2011). PAB are reported to be probiotic having survived in low pH as well as in high bile concentration during GI transit in vitro (Huang and Adams 2004, Suomalainen et al. 2008; Darilmaz and Beyatli 2012) in vivo studies (Huang et al.2003, Suomalainen et al. 2008). PAB showed themselves as growth promoter for intestinal microflora and inhibit common enteric

pathogens (Collado et al. 2007a,b). They possess many other benefits including alleviation of lactose intolerance, antimutagenic activity, anticarcinogenic acitivity and cholesterol lowering effect (Zarate et al. 2002). Propionibacteria have been reported for trehalose accumulation as a result of different adverse growth conditions, such as low temperature, high osmolarity etc (Rolin et al. 1995). Trehalose is well known low calorie sugar synthesized by a wide variety of organisms which could be utilized in replacement of calorie sugars (Neta et al. 2000; Hugenholtz et al. 2002).

2.4.2 Folate production by PAB

PAB are known for their unique, anaerobic, metabolism involving several carbon rearrangement reactions. To catalyse these reactions, PAB contain a wide variety of enzymes with specific cofactors involved in these rearrangements, such as coenzyme B12, folate and biotin (Hugenholtz and smid 2002; Holasova et al. 2004; Hugenschmidt et al. 2010, 2011; Edelmann 2014). These factors make them a promising vitamins producer in applied microbial fermentation industry where *in situ* vitamin production by starter bacteria in fermented foods is performed. PAB show a wide diversity in terms of folate production level with some strains producing as high amounts as reported for high folate producer *S.thermophilus* strains (Hugenholtz et al. 2002).

Only a few studies have reported about folate production by PAB and their results vary considerably (Table 1). The reported levels of folate production varied from 0 to 93 µg/L (Hugenholtz et al. 2002; Hugenschmidt et al.2010, 2011; Van Wyk et al. 2011; Kariluoto et al. 2011), which is similar with production by LAB strains in some of the studies (Sybesma et al. 2003; Kariluoto et al. 2006; Gangadharan et al. 2010; Hugenschmidt et al. 2010, 2011). PAB strains used for the folate investigations reported in Table 1 were isolated from different food and feed sources including meat, cereals, vegetables and dairy products. Most of the PAB strains were tested mainly under low level of oxygen in milk based media which contained lactose or lactate as carbon source. Some PAB strains favoured accumulation of folate inside the cells whereas some excreted folate completely into the medium. According to previous studies, it seems that folate production by PAB is also strain dependant. Further studies by using different carbon sources are needed to understand possible outcome of folate production by PAB strains.

Table 1.Folate production by propionic acid bacteria (Edelmann 2014)

Strains and growth conditions	net folate production Intracellular	extracellular	Method	References
P.freudenreichii ssp. *Shermanii*	0- 93µg/l	-20 to 41µg/l	MA	Hugenholtz et al. 2002
P.thoenii	28 µg/l	8 µg/l	MA	Hugenholtz et al. 2002
P.acidipropionici	58 µg/l	-22 µg/l	MA	Hugenholtz et al. 2002
P.jensenii	51 µg/l	-11 µg/l	MA	Hugenholtz et al. 2002
P.jensenii, P.thoenii pH 5.3, 72 h		≤ 2µg/l	MA	Hugenschmidt et al.2010
P.freudenreichii, Propionibacteria pH (5.5-6.2), 72h, SWP medium		9 µg/l	MA	Hugenschmidt et al.2010
P.acidipropionici (pH 5.0, 72h)		14 µg/l	MA	Hugenschmidt et al.2010
P.freudenreichii pH 5.0, 72h, SWP+pABA		25 µg/l	MA	Hugenschmidt et al.2011
Propionibacterium sp. ABM 5378 Oat matrix, 2 weeks storage	40 ng/g		UHPLC	Kariluoto et al. 2014
P.freudenreichii ssp. Shermanii	7.7 – 32.3 µg/l		HPLC	Van Wyk et al. 2011

2.4.3 Folate production by other bacteria and yeasts

Numerous studies have reported about folate production by LAB strains, bifidobacteria, endogenous bacteria and yeasts (Sybesma et al. 2003; Patring et al. 2005; Hjortmo et al 2005,2008; Kariluoto et al. 2006; Pompie et al. 2007; Gangadharan et al. 2010; Hugenschmidt et al. 2010, 2011; D'Aimmo et al. 2012; Padalino et al. 2012). The ability of bacteria to synthesize folate varies considerably among species. The variation may be due to the lack of genes for biosynthesis of folate, strain differences, and effects of the different culture conditions (Capozzi et al. 2012). Folate production by LAB strains reported in previous studies (Sybesma et al. 2003; Kariluoto et al. 2006; Gangadharan et al. 2010; Hugenschmidt et al. 2010, 2011) were comparatively similar to PAB strains i.e., around 100 µg/l. There was an exceptional result from Hugenschimdt et al. (2010) where *L. plantarum* produced 400 µg/l which was marked as outlier as mentioned by the author and need further investigation to verify the result.

L.lactis was reported to produce up to 256 µg/l when it was genetically manipulated (Sybesma et al. 2003a). Above mentioned studies showed that the growth conditions and strain characteristics affect folate production. In addition, there are strains, for example *L. rhamnosus* and *L. casei*, which did not show folate production under any kind of conditions but instead, they consumed folate (Crittenden et al. 2003; Sybesma et al. 2003b; Herranen et al. 2010; Hugenschmidt et al. 2010). Several different species of bifidobacteria have been screened for their ability to produce folate in low folate or folate free medium. According to previous studies, level of folate production by bifidobacteria species was similar to the levels found in different strains of *S.cerevisiae* and other yeast species (Patring et al. 2005; Hjortmo et al. 2005,2008; Kariluoto et al. 2006; Pompie et al. 2007; D'Aimmo et al. 2012; Padalino et al. 2012). The level of folate production varied from 65 to 90 µg/g dm cell mass. *S. Cerevisiae* itself is a rich source of folate containing 24- 35 µg/g dm of folate (Witthöft et al. 1999; Patring et al. 2005; Patring and Jastrebova 2007). However, it is difficult to compare the level of folate production by bacteria reported by various studies due to the differences in expression of results (per dm or fw; intracellular, extracellular, total folate; weight by weight or weight by volume).

3 EXPERIMENTAL RESEARCH

3.1 Aims

The main objective of this thesis was to study folate production by PAB. The aim was to find promising folate producers among PAB strains which had been investigated for their B12 production ability as a part of the Academy of Finland project- 'Natural fortification of foods: Microbial *in-situ* synthesis of B_{12} and folate in cereal matrices'.This thesis experimental work was divided into two parts: preliminary screening of folate production by 12 strains of PAB and further more detailed study of three selected strains.

In the screening study, 12 PAB strains were studied for folate production under anaerobic condition with lactate as a carbon source and effect of *p*ABA on folate production was also studied. In the second part, folate production by three selected promising folate producers was studied using either glucose or lactate as a carbon source under aerobic fermentation. The metabolic end products of fermentation, organic acids and consumption of carbon substrates were also determined.

3.2 Materials and methods

3.2.1 Chemicals and reagents

All the chemical and reagents used in this experiment are mentioned in this section. Milli-Q water (Millipore Corp., Bedford, Massachusetts, USA) was used in most part of the experiments.

For the preparation of propionic liquid or propionic agar medium (PPA) yeast extract and agar from Becton Dickinson (Franklin Lakes, NJ, USA) were used. Tryptone and DL-sodium lactate 60% w/w from Sigma-Adrich (St.Luis, MO, USA) and NaOH from Merck KGaA (Darmstadt, Germany) were used.

Phosphate buffered saline (PBS) (Dulbecco A tablets, Oxoid Limited, Hamisphere, UK) was used to wash the cells after the fermentation process.

Ches/Hepes, 2-mercaptoethanol, sodium ascorbate were used in the extraction buffer and α-amylase, HK-conjugase, protease were used for enzyme treatment during extraction process of folate.

For the determination of folate, Certified Reference Material BCR 121, wholemeal flour (Institute for Reference Materials and Measurements, Geel, Belgium), 5-

formyltetrahydrofolate as a standard, Folic acid Casei medium and ascorbic acid were used during microbiological assay. Potassium hydroxide, hydrochloric acid and acetic acid were used during pH adjustment.

D-Glucose monohydrate and pyruvic acid from Merck KGaA (Darmstadt, Germany), Sodium DL-lactate solution, succinic acid, propionic acid and sulphuric acid (for mobile phase) from Sigma-Aldrich GmbH (Steinheim, Germany), acetic acid (Riedel-de-Haën, Seelze, Germany) were used for the analysis of organic acids.

3.2.2 Instrumentation and materials

All the instruments and materials used in this experiments are described in this section. 15ml and 50 ml sterile falcon tubes (Sarstedt AG & Co, Nümbrech, Germany) for fermentation process and sample collection, 1.5 mL semi-micro disposable UV cuvettes (12,5 x 12,5 x 45 mm, Brand GmbH, Wertheim, Germany) for the measurement of optical density (OD), sterile plates (Cellstar®, Greiner bio-one GmbH, Frickenhausen, Germany) for propagation of bacteria, anaerobic jars with Anaerocult A strips (Merck KGaA, Germany), Corning Filter System 500 mL (Corning Incorporated, NY, USA) was used to filter glucose solution for preparation of PPA medium with glucose as carbon substrate, 96-well microtiter plate for microbiological assay, 2 mL microcentrifuge tubes (TruCool™, Biocision, Larkspur, CA, USA) for the collection of sugar and organic acid samples, sugar and organic acid samples were filtered with 0.45 µm filters (13 mm, GHP Acrodisc, Pall Life Sciences, Ann Arbor, MI, USA).

12 strains of PAB from the species *P. freudenreichii* and *P. acidipropionici* which had been isolated from dairy and cereal sources and preserved at -80 ºC as glycerol stocks were selected for the determination of folate production in the preliminary screening step. 12 strains of bacteria included two type strains (*P. freudenreichii* subsp. *shermanii* DSM4902 and *P. freudenreichii* subsp. *freudenreichii* DSM20271), four *P. freudenreichii* strains obtained from dairy origin, three *P. freudenreichii* strains obtained from cereal origin and three *P. acidipropionici* (Table 1).

Table 1. 12 strains of PAB used for screening of folate production under anaerobic condition with lactate as carbon source.

Strain no.	Culture	Source
257	*P. freudenreichii*	Dairy origin
259	*P. freudenreichii*	Dairy origin
264	*P. freudenreichii*	Dairy origin
266	*P. freudenreichii*	Dairy origin
275	*P. freudenreichii*	Cereal origin
276	*P. freudenreichii*	Cereal origin
277	*P. freudenreichii*	Cereal origin
278	*P. acidipropionici*	Cereal origin
279	*P. acidipropionici*	Cereal origin
280	*P. acidipropionici*	Cereal origin
281	*P. freudenreichii* subsp. *shermanii* DSM 4902	Type strain
282	*P. freudenreichii* subsp. *freudenreichii* DSM 20271	Type strain

The instruments used in this experiments are given below:

- Analytical balance (Precisa XT 220A, Dietikon, Switzerland)
- Autoclave (Instrulab, Oy Santasalo-Sohlberg Ab, Finland)
- Centrifuge (Centrifuge 5810R, Eppendorf, Hamburg, Germany)
- Linear shaking water bath (Grant Instruments GLS400, Keison products, Essex, UK)
- pH-meter (Meterlab PHM220, Radiometer Analytical, Lyon, France)
- Spectrophotometer (Novaspec II Visible, Pharmacia Biochrom, Cambridge, London)
- Vortex mixer (Fine Vortex, FinePCR, Gyeonggi-do, South Korea)
- Laminar cabinet (BioWizard, KOJAIR TECH OY, Finland)

3.2.3 Cultural conditions for preliminary screening of folate production (with and without para-aminobenzoic acid in anaerobic conditions)

Glycerol stocks of strains preserved at -80 °C were used to propagate culture as starting inoculums by streak plate method. The strains were grown on the PPA medium composed of 5.0 g tryptone (Sigma-Aldrich), 10.0 g yeast extract (DIFCO Becton, Dickinson), 14.0 ml 60% w/w DL-sodium lactate (Sigma-Aldrich) per litre with pH adjusted to 6.7 prior to autoclaving at 121°C for 15 minutes. For the solid medium 1.5% of agar was added and pH adjusted to 7.3. MilliQ water was used to prepare the medium.

In the screening step, all the cultivations were carried out under anaerobic conditions, since PAB strains were observed to grow better without oxygen in studies on vitamin B12 production. The strains were incubated anaerobically at 30 ºC first for 96 hours on agar plates, then for 72 hours in 10 ml pre-cultures in PPA broth in 15 ml Falcon tubes, in triplicate, and finally in 50 ml PPA in 50 ml falcon tubes, in triplicate, with and without addition of (2mg/ L) (*p*ABA) and grown for 96 hours. As exception for above mentioned process, strain 276 was incubated in aerobic condition instead of anaerobic cultivation due to lack of growth without oxygen. In addition, PPA broth without any inoculation (with and without addition of *p*ABA) were also incubated as control samples. Air tight plastic jars were used to keep inoculated agar plates and broth tubes and anaerokult sachets (Anaerokult, Merck, Germany) were kept inside jars to maintain anaerobic condition. The optical density (OD) of the pre-cultures was measured at 600 nm (Novespec® II, Amersham Pharmacia Biotech, USA) in order to normalise the inoculum level in the final cultures to OD_{600}= 0.01.

OD of all the samples were measured after incubation. The cell mass and supernatant were separated by centrifugation. Samples were centrifuged (Centrifuge 5810R, Eppendorf, Hamburg, Germany) at 10,000 rcf for 5 minutes at 4 ºC. pH of the supernatants were measured. Tubes with cell pellet were washed with phosphate buffer solution (PBS) to remove remaining PPA from cell mass and centrifuged again to get net cell pellet. The tubes with cell pellet were weighed and deduced with previously weighed tubes to determine the cell mass. The collected samples were stored in freezer at -20 ºC until determination of folate.

3.2.4 Growth conditions in further studies with three selected strains

Based on the folate results of the preliminary screening, three strains (257, 277, 281) were selected to study their folate production either with lactate or glucose as a carbon source. Furthermore, anaerobic cultivation was changed to aerobic, to investigate the effect of normal oxygen level on growth and folate synthesis of selected PAB strains. Generally, food

applications of fermentation microbes involve aerobic environment. Two types of medium were prepared (normal PPA and PPA with glucose) for cultivations. The composition of normal PPA medium was similar as mentioned in section 3.2.3 but PPA with glucose was prepared by replacing lactate with equimolar amount of glucose i.e, glucose solution with final concentration 0.67 % w/v (APPENDIX 1) after autoclaving. Glucose solution was sterilized with microfiltration (0.22 μm) using Corning INC, Corning, NY, USA. pH of media was adjusted to 6.7 before autoclaving. Sterile filtered glucose solution was added to medium without lactate before inoculation of bacterial inoculum. Selected strains were grown in the two media and growth curve of all the strains were studied to determine the harvesting time points during four different growth phases.

3.2.5 Growth curves

The growth curves of three strains in two media were studied to determine the optimal harvesting time points for four different growth phases i.e., mid-exponential, early stationary, late staionary and resting phase. Selected bacterial strains were plated and incubated as described in section 3.2.3 except culture tubes were incubated aerobically as our further study with these strains was to study folate production in aerobic condition. Tubes were incubated aerobically at 30 ºC and OD of all the tubes was measured every second hours after 24 hours of incubation on the basis of previous growth rate observation reported in MSc thesis by Sangsuwan (2013).

3.2.6 Fermentation and cell harvest

Three selected strains were fermented aerobically in two conditions as stated above in section 3.2.4 and samples were taken at four growth phases as explained in section 3.2.5. Each strain was grown separately for four different allocated growth phases in triplicate. 100 ml of medium were prepared for the first harvesting time point (mid-exponential phase) instead of 50 ml to ensure sufficient cell mass for later folate determination. Additionally, four falcon tubes with both types of medium were incubated without inoculated cells as controls. Cells were harvested at four harvesting time points according to four different growth phase allocated during growth curve determination and a control tube was also taken out of incubation at each time point to see the effect of incubation on folate in medium. Samples were collected and stored similarly as detailed in section 3.2.3.

3.2.7 Determination of folate

Extraction

Extraction of folate was carried out always under subdued light to avoid possible degradation or loss of folate by visible light. CHES/HEPES extraction buffer was used for the heat extraction. Appendix 2 shows the composition of the extraction buffer. 2 ml of supernatant and approximately 0.1 g of cells (depending upon the cell mass production) were weighed in Pyrex tubes. 12 ml of extraction buffer was added into each tube and into an empty tube considered as blank sample after which the tubes were flushed with nitrogen and then kept in boiling water for 10 minutes.

One gram of control sample (Certified Reference Material BCR 121, wholemeal flour, Institute for Reference Materials and Measurements, Geel, Belgium) was also weighed in Pyrex tube and analyzed similarly as other samples.

Tri-enzyme treatment

Extracted samples were adjusted to pH 4.9 and 1 ml of α-amylase (20mg/ml) and 1ml of HK-conjugase were added consecutively in each sample, which were then flushed with nitrogen and kept in shaking water bath for incubation at 37 ºC for 3 hour. After incubation pH was adjusted to pH 7.0 and 2ml of protease (2mg/ml) was added. Samples were flushed with nitrogen and kept for incubation in shaking water bath at 37ºC for 1 hour. After incubation samples were boiled in boiling water bath for 5 min to deactivate the enzyme activity and cooled on ice. All the samples were filled to an exact volume of 25 ml with 0.5% sodium ascorbate (pH 6.1). Then two dilutions for each sample were prepared with 0.5% sodium ascorbate (pH 6.1) in a 10ml volumetric flask along with blank sample with similar dilutions.

Folate determination

The total folate content was determined with microbiological method (MBA). Standard solution was prepared from 5-formyltetrahydrofolate with 0.5% sodium ascorbate, pH 6.1. Assay organism was prepared by mixing 1 ml of thawed glycerol- cryopreserved *Lactobacillus rhamnosus* ATCC 7469 with 2.5 ml of sterile saline in a sterile plastic tube and 150µl of it was mixed in assay medium. Appendix 3 shows the flow chart of preparation of assay medium.

MBA was performed on a 96- well microtiter plate. Standard solution (calibrant) from 0 to 80 pg/well and sample extracts, both 100 µl, were inoculated into wells of microtiter plate (4 wells for each concentration). 200 µl of prepared assay organism was inoculated into each well with

multi-channel pipette. The plate was covered, set into plastic container and incubated at 35ºC for 18 hours.

After 18 hours of incubation, turbidity of the wells was measured at 595 nm ((MTX Lab Systems, Inc., Vienna, Virginia, USA.). Results were calculated with appropriate blank reduction and dilution factors. Folate content of the fermentation replicate was calculated as per gram fresh cell biomass (ng/g), per milliliter of medium (ng/ml) and total net folate per litre (µg/l). Averages and standard deviation were calculated using three biological replicates.

3.2.8 Analysis of organic acids and sugars

Preparation of samples and standard solution

Frozen supernatants from four different growth phases were diluted 1:10 with Milli-Q water and vortexed for 5 sec. Standard solution mixture containing glucose, lactate, propionate, acetate, pyruvate and succinate was prepared with concentration of 1 mg/ml of each compound and diluted 1:10 and 1:25 with Milli-Q water. Standard solution and samples were filtered with 0.45 µm filter (Acrodisc, Pall Corporation, USA) into HPLC vials prior to HPLC analysis.

The HPLC system consisted of an Aminex HPX-87H column (300 x 7.8 mm, hydrogen form, 9 µm; Bio-Rad Laboratories Inc, California, USA), Waters 515 HPLC pump with online degassing, Waters 717 auto sampler, and Waters 487 dual λ UV (Ultraviolet) absorbance detector (Waters, Milford, Massachusetts, USA), and HP 1047A RI (Refractive Index) detector (Hewlett-Packard 46 Company, California, USA). RI detector was used to detect sugars whereas organic acids were detected with a UV detector (210 nm). The mobile phase was 10 mM H_2SO_4 with flow rate 0.6 ml/min. Samples were injected once with volume of 60 µl. Sugar and acid concentrations were quantitated by using a multilevel external calibration curve ranging from 0.8 to 40 ng and dilution factors were taken into account during calculations.

3.2.9 Quality assurance of folate determination

Certified reference material BCR 121 (wholemeal flour, Institute for Reference Materials and Measurements, Geel, Belgium) was analysed in each incubation as quality control sample (section 3.2.7). Action limits in the control charts were 500±70 ng/g dm, which is the certified value for BCR 121. The results of the set analyses were rejected if the total folate content of the BCR 121 was outside the action limits.

3.2.10 Statistical analysis

Statistical analysis was carried out using IBM SPSS program version 20.0 (IBM Corporation, NY, USA). Variables were tested using two-tailed Independent test. A p-value of < 0.05 was considered statistically significant. Averages and standard deviation (SD) were calculated using Microsoft Office Excel 2010 (Microsoft Corporation, Washington, USA).

3.3 Results

3.3.1 Preliminary screening

3.3.1.1 Growth and pH during fermentation

The results of growth and pH after 96– hour fermentation with and without added *p*ABA are shown in Table 1. Strain 279 and 280 reached the highest OD values (3.59-3.95) with and without *p*ABA whereas strain 276 had the lowest OD value (0.35) when it grew with *p*ABA. Addition of *p*ABA had no effect on the growth of the strains during the 96– hour fermentation. Only the growth of strain 280 was significantly higher ($p< 0.05$) without *p*ABA than with it (Appendix 1). OD values of all remaining strains were within the range of 1.67- 2.61.

The initial pH of both types of medium was 6.51. Strain 278 dropped its pH to 5.81 which was the lowest value obtained. The pH obtained from cultivations of the strains were within the range of 5.81-6.38 and *p*ABA did not show effect on pH ($p> 0.05$).

Table 1. OD value (600 nm) and pH of 12 PAB strains after 96 hour fermentation in PPA medium without and with added *p*ABA. Results are means of three biological replicates. SD=Standard deviation; PPA= Propionic acid medium; PPA+*p*ABA= *p*ABA added PPA medium. (*) indicate the significant difference between two variables at $p< 0.05$.

Strain	OD PPA	SD	pH	OD PPA+*p*ABA	SD	pH
257	2.19	0.03	6.16	2.06	0.08	6.14
259	2.57	0.05	6.18	2.6	0.04	6.24
264	2.19	0.29	6.19	2.21	0.3	6.19
266	1.94	0.01	6.11	1.96	0.07	6.10
275	0.37	0.01	6.38	0.35	0.01	6.37
276	1.86	0.08	6.23	1.67	0.19	6.21
277	2.61	0.04	6.12	2.54	0.07	6.13
278	2.37	0.51	5.81	2.12	0.24	5.81
279	3.68	0.1	6.01	3.59	0.08	5.98
280	3.95*	0.01	6	3.6*	0.01	6
281	2.01	0.00	6.22	1.9	0.01	6.22
282	2.17	0.01	6.14	2.16	0.03	6.19
Control			6.51			6.51

3.3.1.2 Cell mass produced during fermentation

The produced cell masses (g,fw) by the strains during fermentation are presented in Figure 1. Strain 266 acquired the highest cell mass production (0.66 g) in normal PPA and (0.64 g) in PPA with added *p*ABA. Strain 275 produced the lowest cell mass with (0.02- 0.03 g) in normal PPA and in PPA with added *p*ABA. The produced cell masses after 96 hour fermentation by other strains ranged from 0.19 to 0.35 g. Addition of *p*ABA did not effect on cell mass production of the strains significantly ($p > 0.05$). By contrast strain 281 showed significantly ($p < 0.05$) higher cell mass production without than with *p*ABA addition (Appendix 1). The produced cell mass by strain 281 was 0.27 g in normal PPA while 0.25 g in PPA with added *p*ABA.

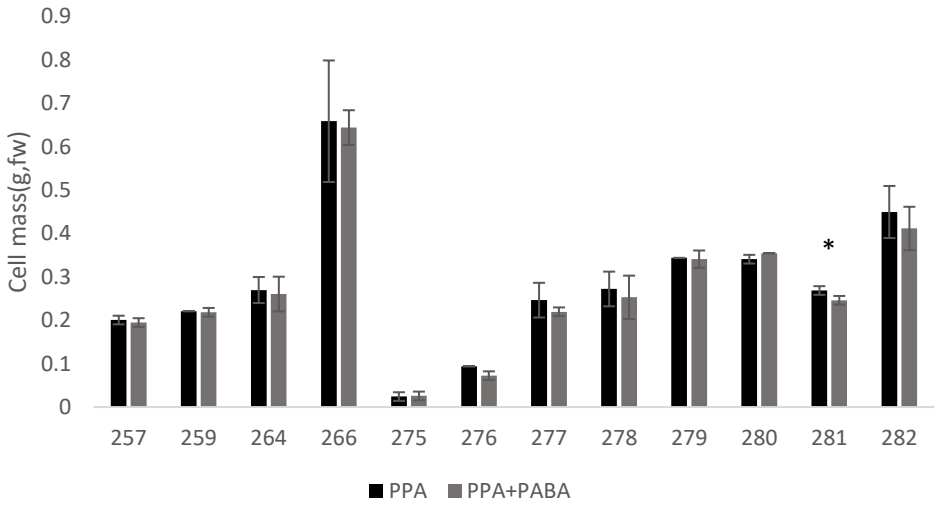


Figure 1. Cell mass (g,fw) produced by propionic acid bacteria strains in PPA medium without and with added *p*ABA after 96 hour fermentation in anaerobic condition. (Strain 276 was in aerobic condition). Results are means of three biological replicates and error bars represent standard deviation. (*) indicate significant difference between two variables at $p < 0.05$.

3.3.1.3 Folate production

The extracellular net folate contents are presented in Figure 2a. Overall, excretion of folate into medium ranged from 1 ng/ml to 107 ng/ml. Strains 257 and 277 excreted folate into the culture medium more than other strains. The net concentrations were 107 ng/ml and 61 ng/ml with addition of *p*ABA and 56 ng/ml and 83 ng/ml without addition, respectively. In supernatants of the strain 278 and 282, folate contents were lower than originally in PPA. Strain 259 with added pABA excreted 10 ng/ml folate into medium while doubled the amount of folate excretion in normal PPA (18 ng/ml). Strain 276 excreted folate slightly into the normal PPA medium (1 ng/ml) while excreting 40 fold more into the medium with *p*ABA addition.

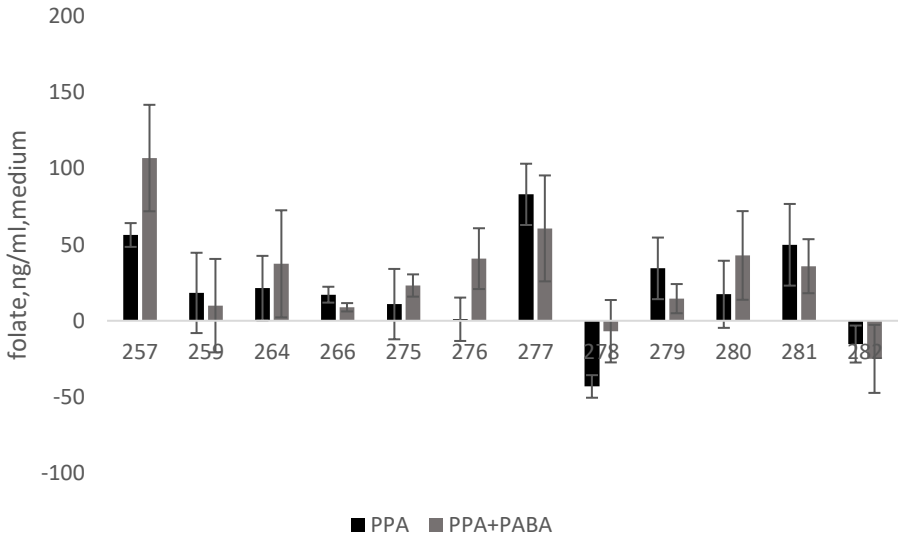


Figure 2a. Net extracellular folate content (folate content in medium deducted from folate content in control medium) in PPA medium without and with added *p*ABA after 96 hour fermentation. Results are means of three biological replicates and error bars represent standard deviation.

The intracellular folate contents were within the range of 3601 – 28954 ng/g cells (Figure 2b). Strain 275 accumulated the highest amount of folate 28954 ng/g in normal PPA whereas folate concentration of the strain 266 was the lowest 3601 ng/g in PPA with added *p*ABA.

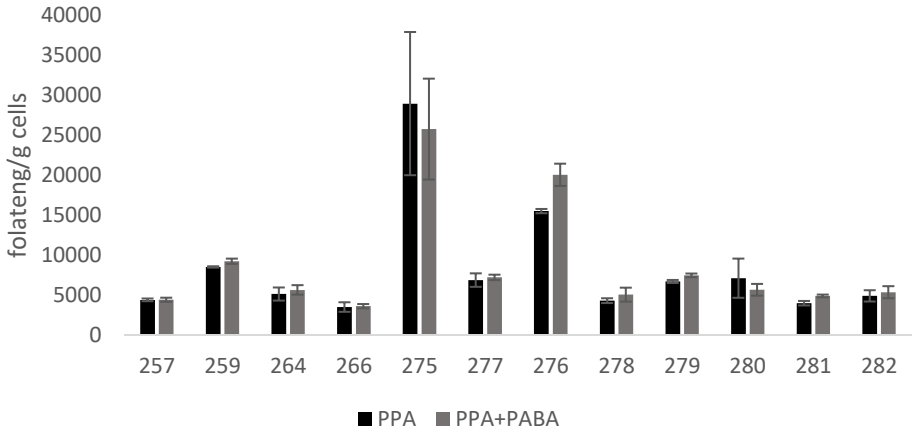


Figure 2b. Intracellular folate production by propionic acid bacteria strains in PPA medium without and with added *p*ABA after 96 hour fermentation. Results are means of three biological replicates and error bars represent standard deviation.

Figure 2c shows the total net folate production of 12 PAB strains without and with *p*ABA addition.

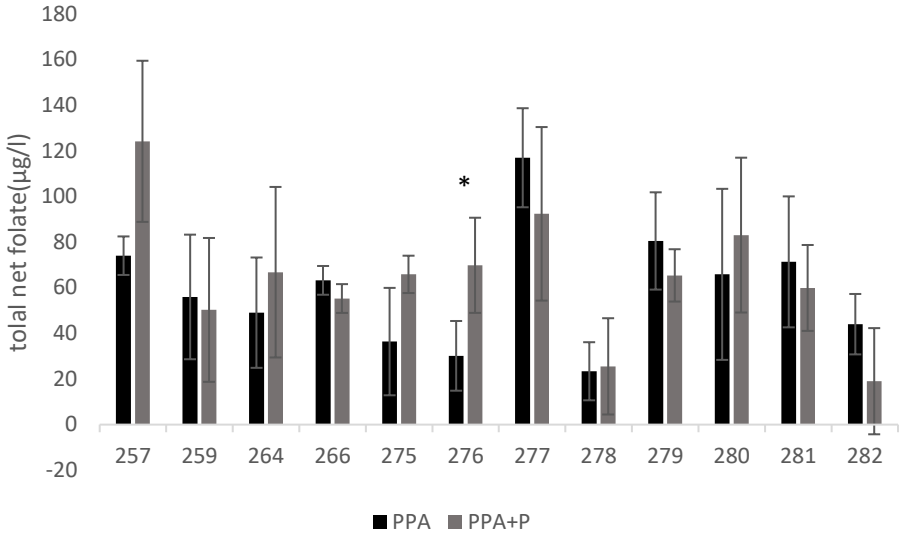


Figure 2c. Total net folate production (in cell mass and medium) by propionic acid bacteria strains in PPA medium without and with added PABA during 96 hour fermentation. Results are means of three biological replicates and error bars represent standard deviation. (*) indicate significant difference between two variables at $p < 0.05$.

When the folate production in the cells was combined to amount of folate excreted out from the cells, it was examined as total net folate production after fermentation. Thus, the net folate production ranged from 19- 124 µg/l. Strain 257 and 277 had the highest total net folate production 124 µg/l with added *p*ABA and 117 µg/l in normal PPA respectively. Strain 282 yielded the lowest folate content with added *p*ABA accounting 19 µg/l.

Strain 276 produced significantly higher folate with *p*ABA addition than in normal PPA ($p < 0.05$). By contrast, with *p*ABA addition, folate production by strain 282 was clearly lower than without it. Other strains did not show significant difference on net folate production between with and without pABA addition ($p > 0.05$, Appendix 1).

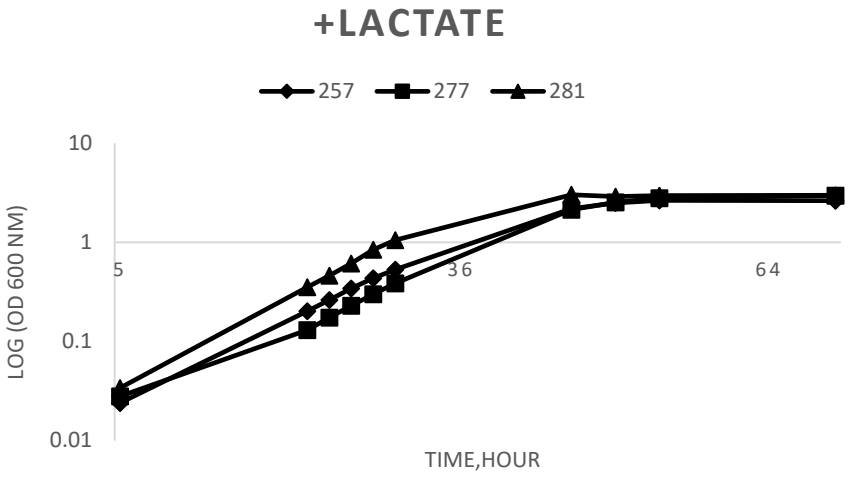
3.3.2 Further studies with three selected strains

3.3.2.1 Growth curves and harvesting times

Based on the folate production in screening part, three promising folate producers (257,277,281) were selected for further studies. To follow their folate production at four different growth phases (mid-exponential, early stationary, late stationary and resting phase)

in aerobic condition either with lactate or glucose as carbon source, growth curves for strains were determined. The growth curves of three strains with lactate or glucose as carbon source are illustrated in Figure 3.

A



B

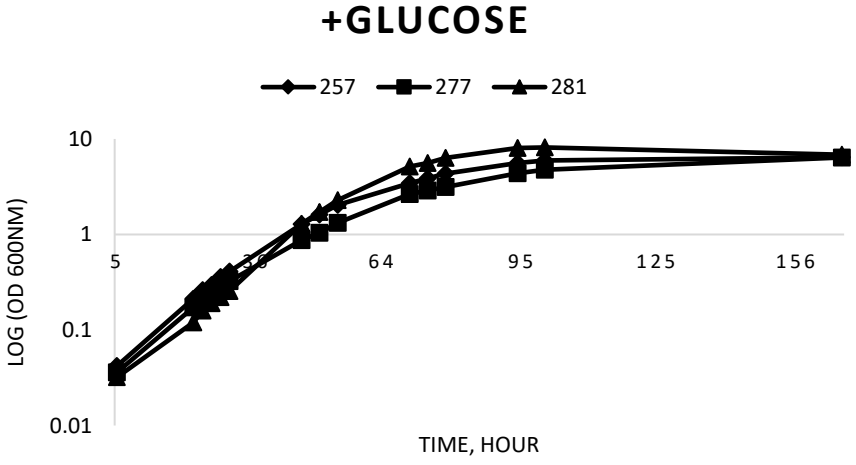


Figure 3. Growth curves of strains 257, 277, 281 with lactate (**A**) and glucose (**B**) as a carbon source

The growth of strains was faster and reached their stationary phases quicker with lactate than with glucose as carbon source. With glucose as carbon source, strains had lengthy growth phases and gradual growth was observed until the resting phase. Based on the growth curves with lactate and glucose- four harvesting time points for strains were selected (Table 2).

Table 2. Growth phases with two different carbon sources and their respective harvesting time points. +L= strains grown with lactate as carbon source; +G= strains grown with glucose as carbon source.

Growth phase	Harvesting time after inoclulation (+L)	Harvesting time point after inoculation (+G)
Mid-exponential	24 h	46 h
Early stationary	48 h	96 h
Late stationary	96 h	144 h
Resting	168 h	216 h

3.3.2.2 Growth and pH during fermentation

OD values and pH at four different growth phases with two different carbon sources are presented in Table 3. Strains grown with glucose obtained higher OD values than with lactate. With glucose, strains 257 and 277 reached their highest OD values 4.87 and 4.21, respectively at resting phase while strain 281 reached the highest OD value 7.45 at late stationary phase. After that OD value decreased at resting phase. Strain 257 and 277 obtained their highest OD values 2.24 and 2.74, respectively, at late stationary phase while strain 281 acquired its highest OD value 2.80 at early stationary phase.

Table 3. OD values (600 nm) and pH of three strains (257,277,281) at four different growth phases (T1= mid-exponential; T2= early stationary; T3= late stationary; T4= resting phase) with two different carbon source during aerobic fermentation. Results are means of three biological replicates. SD=Standard deviation; +G=culture grown with glucose as carbon source; +L=culture grown with lactate as carbon source

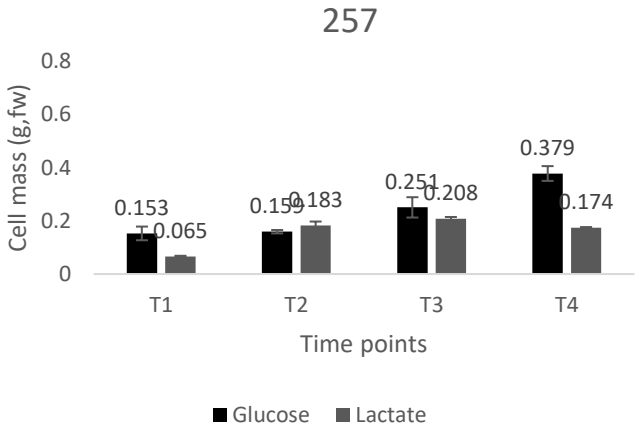
Strain	Time point	OD (+G)	SD (+G)	pH (+G)	OD (+L)	SD (+L)	pH (+L)
	T1	0.57	0.03	5.96	0.24	0.01	6.23
257	T2	1.97	0.04	5.17	1.78	0.01	6.07
	T3	3.49	0.43	4.89	2.24	0.01	6.22
	T4	4.87	0.11	4.58	1.61	0.02	6.26
	T1	0.38	0.03	6.08	0.24	0.01	6.19
277	T2	1.01	0.13	5.52	2.53	0.02	6.21
	T3	1.82	0.11	5.17	2.74	0.02	6.39
	T4	4.21	0.14	4.75	2.29	0.04	6.23
	T1	0.74	0.01	5.86	0.39	0.04	6.16
281	T2	4.55	0.33	4.70	2.80	0.05	6.32
	T3	7.45	0.31	4.46	2.48	0.00	6.28
	T4	6.14	0.04	4.41	1.63	0.03	6.25
Control				6.51			6.23

pH of strains with lactate stayed more stable while pH of strains with glucose media dropped continuously until resting phase. pH of strain 281 with glucose dropped to the lowest 4.41 at resting phase.

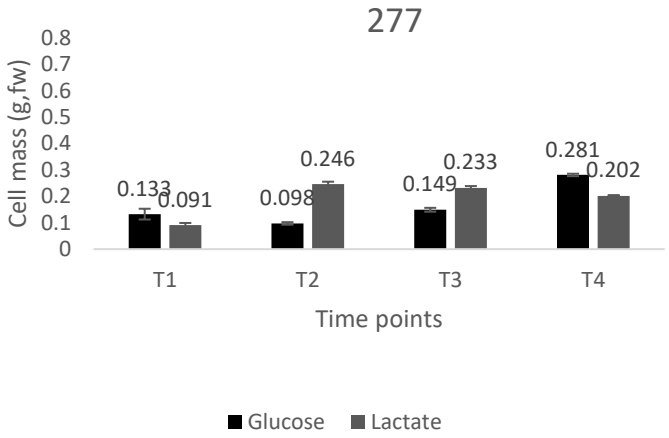
3.3.2.3 Cell mass production

The produced cell masses at different growth phases with either glucose or lactate as carbon source is illustrated in Figure 4 (**A**, **B**, **C**). Cell mass production was mostly higher with glucose than with lactate. With glucose, there was a trend of increment in cell masses up to resting phase while with lactate, cell mass production increased upto late stationary phase and decreased down at resting phase.

A



B



C

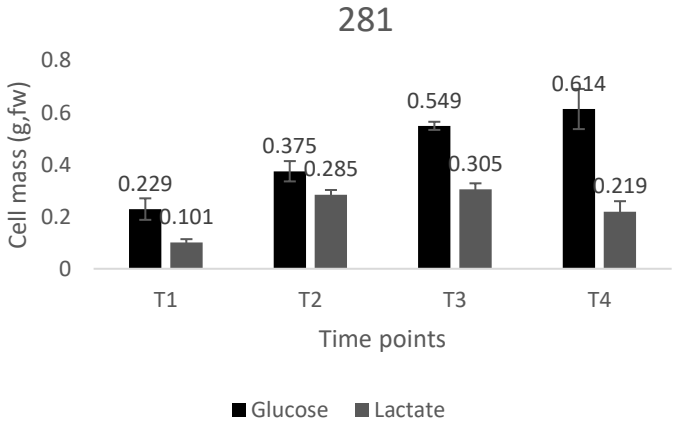


Figure 4. Cell masses (g,fw) produced by strains 257(**A**),277(**B**),281(**C**) in PPA medium with glucose and lactate as carbon source during four different growth phases (T1= mid-exponential; T2= early stationary; T3= late stationary; T4= resting phase) during aerobic fermentation. Results are means of three biological replicates and error bars represent standard deviation.

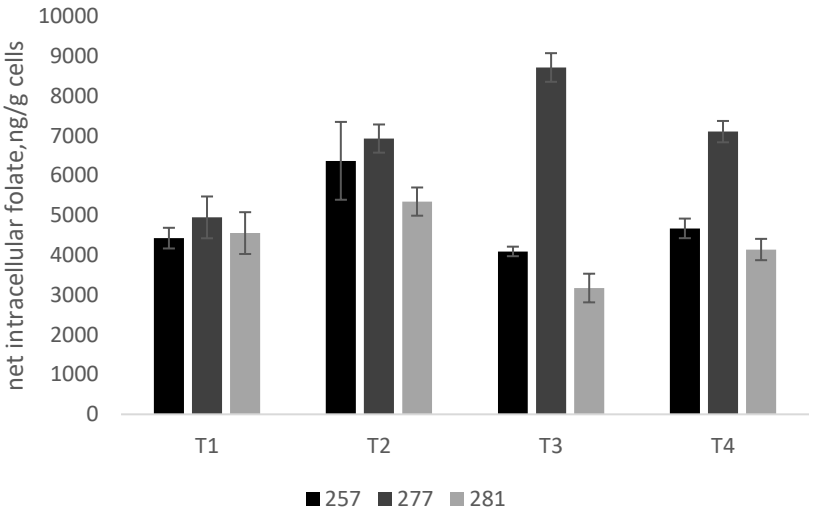
Strain 281 yielded the highest cell mass 0.61 g at the resting phase with glucose and 0.30 g at late stationary phase with lactate. Strain 257 produced the lowest cell mass (0.065 g) with lactate at mid-exponential phase while lowest cell mass production with glucose was 0.091 g at early stationary phase by strain 277.

3.3.2.4 Folate production

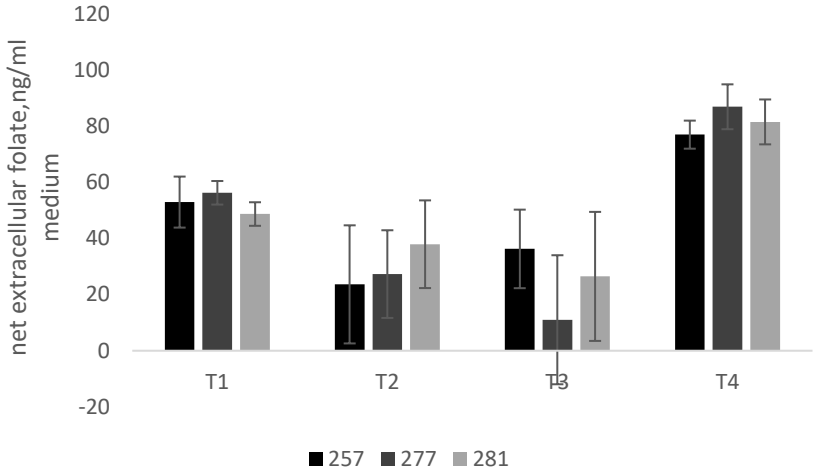
Folate production with lactate as a carbon source

Intracellular, extracellular and total net folate contents in media with lactate at different growth phases are presented in Figure 5. At early stationary phase, intracellular folate production by strains 257 and 281 was at the highest, while strain 277 yielded the highest intracellular folate concentration at late stationary phase (Figure 5A). 277 yielded the highest intracellular folate (8715 ng/g) at late stationary phase and strain 257 and 281 produced the highest intracellular folate 6370 ng/g and 5346 ng/g, respectively at early stationary phase.

A



B



C

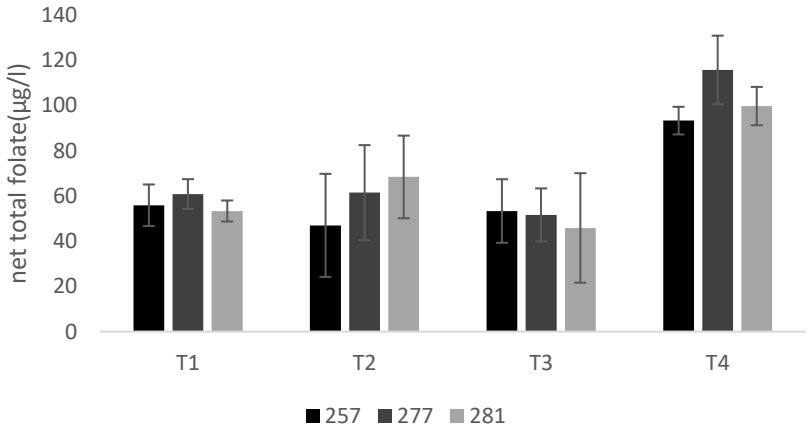


Figure 5. Folate production by strains 257,277,281 [A: ng/g cells, B: ng/ml medium (folate content of medium deducted from folate content of control medium) and C: total folate µg/l] in PPA medium with lactate as carbon source at four different growth phases (T1= mid-exponential; T2= early stationary; T3= late stationary; T4= resting phase) during aerobic fermentation. Results are means of three biological replicates and error bars represent standard deviation.

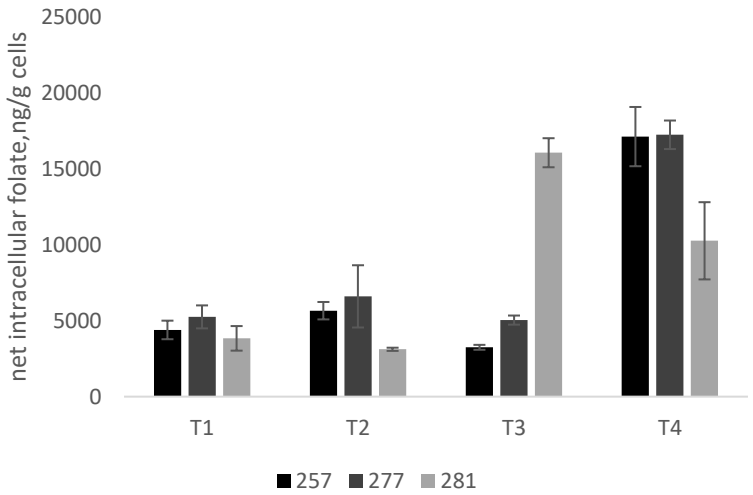
Strains released notable amount of folate into the medium. Strains excreted the highest amount of folate at resting into the medium. The level of excretion of folate into the medium was lower at stationary phases by all the strains. Strains 257, 277 and 281 excreted 77, and 87 and 82 ng/ml of folate into medium, respectively (Figure 5B). The net total folate production by strains

257, 277 and 281 were the highest at resting phase accounting 93, 116 and 100 µg/l, respectively (Figure 5C).

Folate production with glucose as a carbon source

Intracellular, extracellular and total net folate contents in media with glucose at different growth phases are shown in Figure 6. Strain 281 had the lowest intracellular folate accumulation at early stationary phase and the highest (16047 ng/g) at late stationary phase. Strain 257 and 277 had the highest intracellular folate accumulation (17109 ng/g and 17227 ng/g, respectively) at the resting phase (Figure 6A). The folate was excreted in notable amount into medium at the mid-exponential phase and the highest amount of folate was excreted at the resting phase. At stationary phase there were very low level of folate excretion into medium or no excretion at all. The highest extracellular folate contents for strains 257, 277 and 281 in medium were 76, 56 and 61 ng/ml, respectively, at resting phase. The net total folate production of strains 257, 277 and 281 were the highest at resting phase with 113, 92 and 88 µg/l respectively.

A



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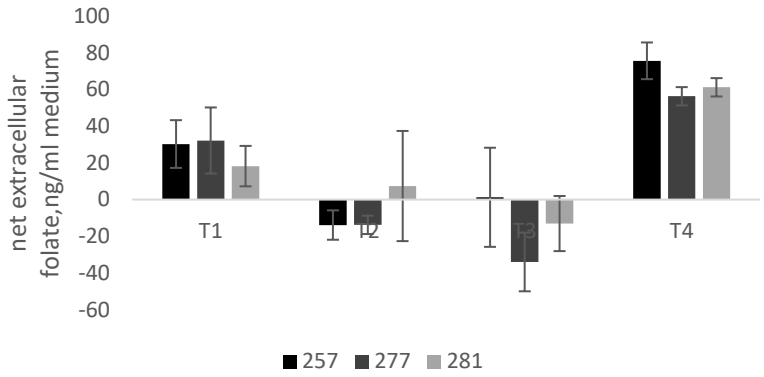

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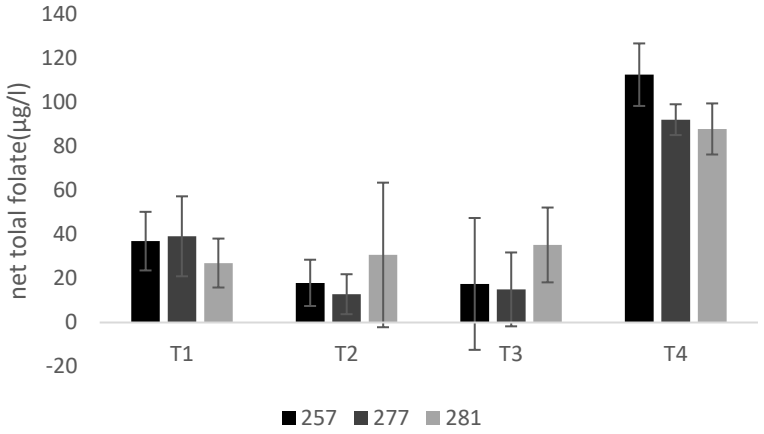


Figure 6. Folate production by strains 257,277,281 [A: ng/g cells, B: ng/ml medium (folate content in medium deducted from folate content of control medium) and C: total folate µg/l] in PPA medium with glucose as carbon source at four different growth phases (T1= mid-exponential; T2= early stationary; T3= late stationary; T4= resting phase) during aerobic fermentation. Results are means of three biological replicates and error bars represent standard deviation.

Net total folate production of three strains with different cultivation condition at stationary phase

When folate production results of strains 257, 277 and 281 in the screening step after 96 hour fermentation are also taken into consideration, all three strains produced their highest net folate in anaerobic cultivation with lactate as carbon source (Figure 7). In the further study step, aerobic cultivation did not increase the folate production. In addition, strains produced less folate with glucose in aerobic cultivation than with lactate in most of the samples.

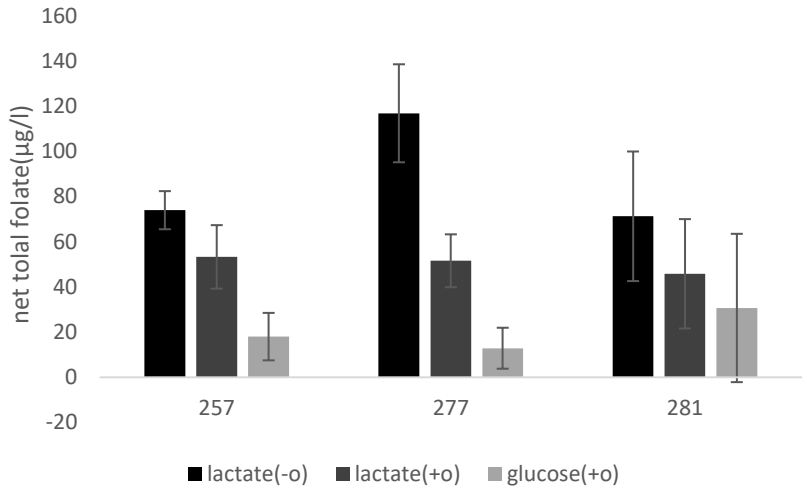


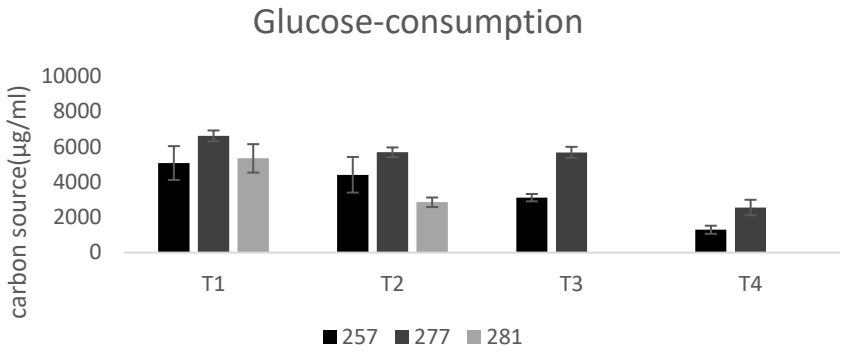
Figure 7. Net total folate production by strains 257,277,281 with three different cultivation conditions at stationary phase. Lactate (-o)= Anaerobic cultivation with lactate as carbon source; lactate (+o)= Aerobic cultivation with lactate as carbon source; glucose (+o)= Aerobic cultivation with glucose as carbon source. Results are means of three biological replicates and error bars represent standard deviation.

3.3.2.5 Carbon source consumption and organic acid production

Carbon source consumption by strains is illustrated in Figure 8. The initial amount of the glucose was 6706 µg/ml. Strain 257 utilized half of the glucose until it reached the late stationary phase and 1293 µg/ml glucose was left in the medium at resting phase. Strain 277 consumed the glucose substrate very slowly. There was still 5686 µg/ml of glucose left at late stationary phase. The consumption of substrate increased at resting phase and glucose decreased to 2558 µg/ml which remain unutilized in the medium. Strain 281 catabolized all glucose by the end of early stationary phase.

The strains consumed lactate effectively at the beginning of the fermentation. The initial amount of lactate was 10533 µg/ml. Strain 281 utilized lactate completely already during mid-exponential phase, while strain 257 and 277 consumed it completely by the end of the early stationary phase.

A



B

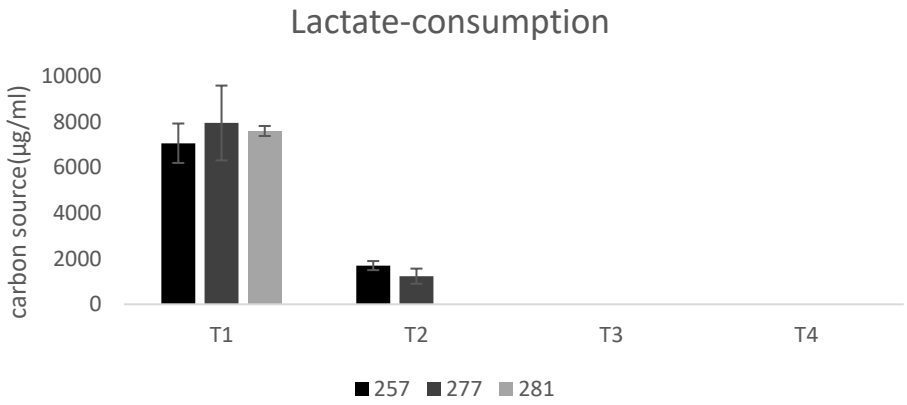


Figure 8. The content of unconsumed carbon sources (glucose A and lactate B) in the medium at four different growth phases (T1= mid-exponential; T2= early stationary; T3= late stationary; T4= resting phase) by strains 257, 277 and 281 during aerobic fermentation. Results are means of three biological replicates and error bars represent standard deviation.

Organic acid production

Metabolic end products, such as propionate, acetate and intermediate metabolic product pyruvate were determined in the cell free supernatants. Strains produced more metabolic end product with lactate than with glucose.

Acid production with glucose in aerobic fermentation

The concentration of acetate (1191 µg/ml) and propionate (3215 µg/ml) produced by strain 281 were the highest with glucose at resting phase and though strain 277 started to produce organic acids early at mid-exponential phase, it produced the lowest acetate and propionate (Figure 9).

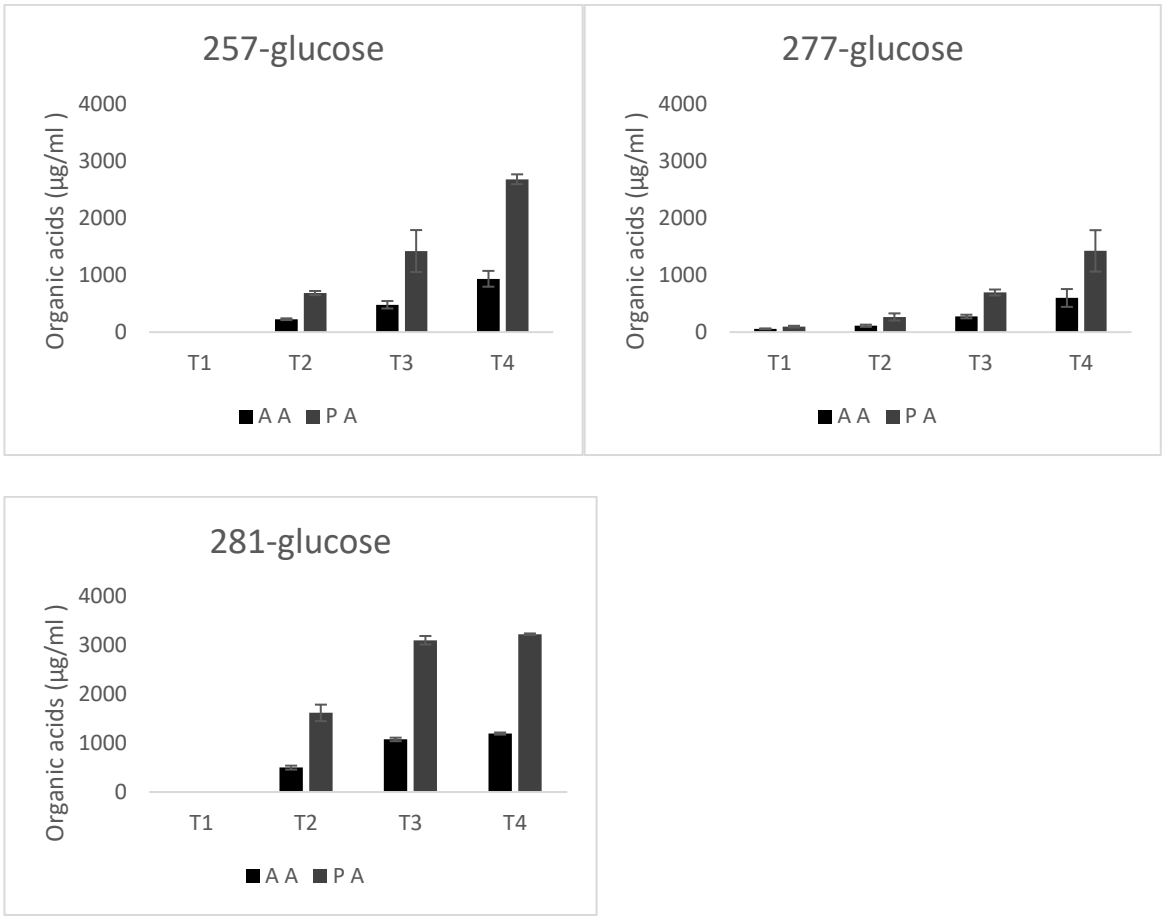


Figure 9. Organic acid production by strains 257,277 in PPA medium with glucose as carbon source at four different growth phases (T1= mid-exponential; T2= early stationary; T3= late stationary; T4= resting phase) during aerobic fermentation. Results are means of three biological replicates and error bars represent standard deviation.

Acid production with lactate in aerobic fermentation

With lactate strain 257 produced the highest concentration of acetate and propionate (2228 µg/ml and 5584 µg/ml, respectively) at resting phase even though it started to produce after mid-exponential phase. Production of pyruvate was detected at mid-exponential phase in all three strains (Figure 10).

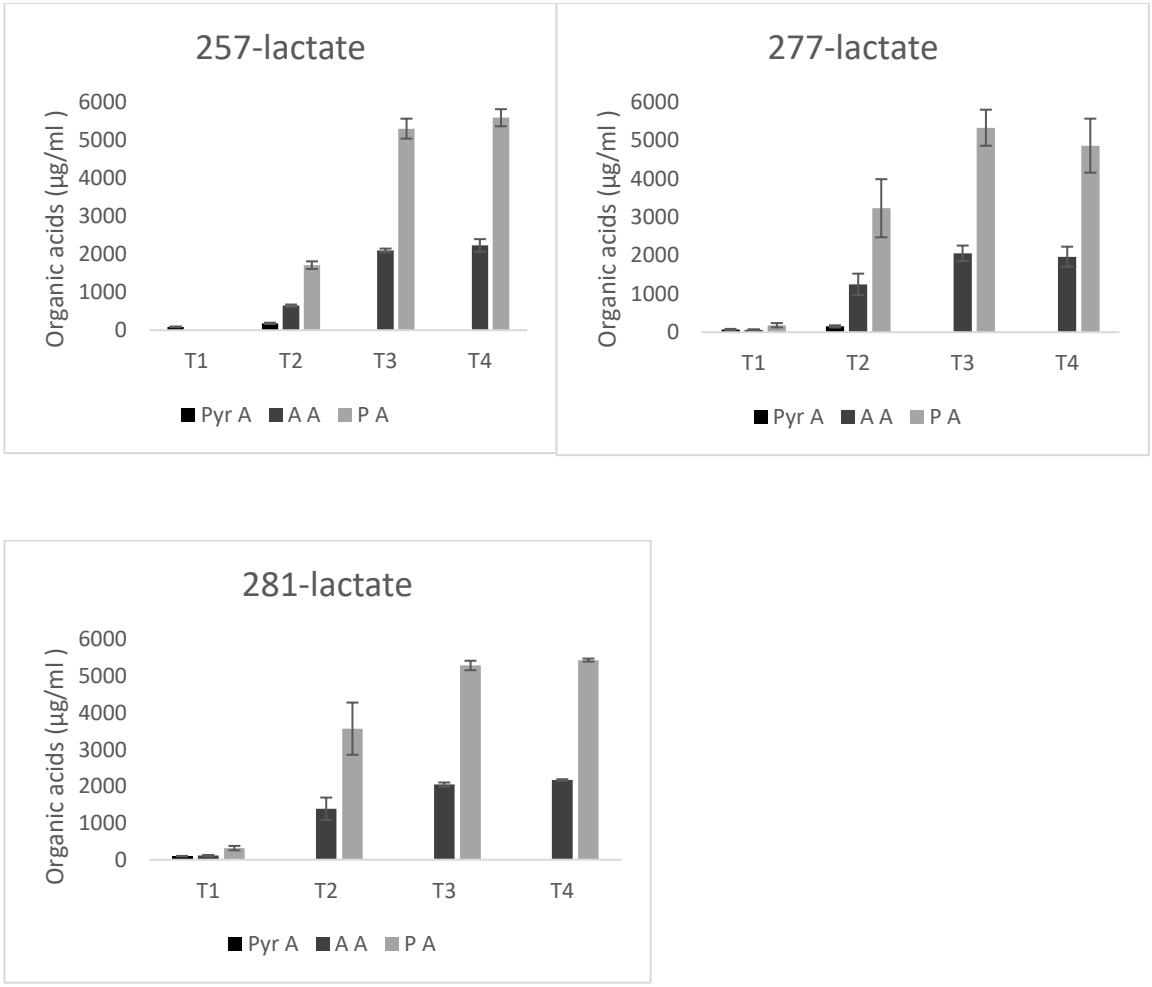


Figure 10. Organic acid production by strains 257,277,281 with lactate as carbon source at four different growth phases (T1= mid-exponential; T2= early stationary; T3= late stationary; T4= resting phase) during aerobic fermentation. Results are means of three biological replicates and error bars represent standard deviation.

Acid produced by strains 257, 277 and 281 after 96 hour anaerobic fermentation

When results of acid production by strains 257, 277 and 281 in screening step after 96 hour anaerobic fermentation taken into consideration, the concentration of acetate and propionate produced by strain 277 were the highest amounting 1902 µg/ml and 4993 µg/ml, respectively (Figure 11).

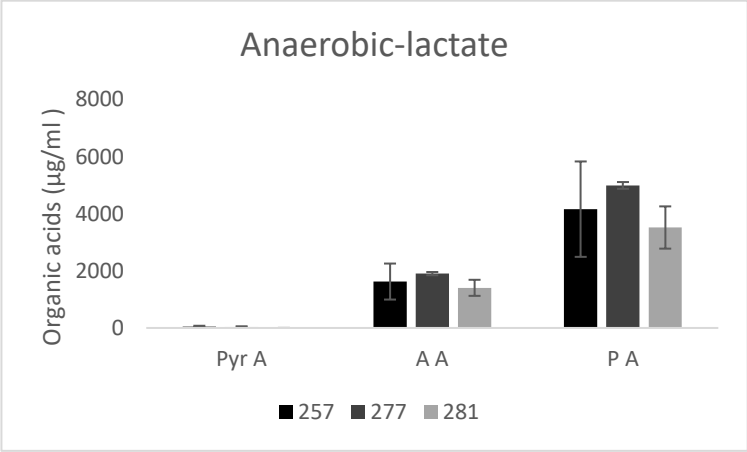


Figure 11. Organic acid production by strains 257,277,281 with lactate as carbon source after 96 hour anaerobic fermentation. Results are means of three biological replicates and error bars represent standard deviation.

Aerobic fermentation with lactate was more promising for acid production than anaerobic condition when acid production after 96 hour fermentation in screening step was taken into consideration. The intermediate metabolic product, pyruvate was detected with lactate during exponential phase.

3.4 Discussion

3.4.1 Preliminary screening

12 PAB strains from dairy and cereal sources were screened for their folate production in anaerobic fermentation and lactate as carbon source. To examine the effect of *p*ABA, a precursor of folate biosynthesis, on folate production, strains were grown in medium with addition of *p*ABA as well. In the course of studying effect of *p*ABA on folate production, effect of *p*ABA on growth, pH, cell mass was also examined.

3.4.1.1 pH, growth and cell mass production

pH

The addition of *p*ABA had no effect on acid production or on folate synthesis as pH value of normal PPA medium and folate production remained similar to that without *p*ABA addition. The pH of the medium did not drop drastically for any of the strains with lactate as a carbon source in the screening step. pH of all the strains remained close to the pH value of control medium (6.5). As mentioned in MSc thesis by Hiippala (2014), lactate is a conjugate base of a weak acid (lactic acid) and, thus, it can act as a buffer as well, which could be the reason for the obtained results. Strains of *P. acidipropionici* (278, 279,280) dropped their pH to the lowest among 12 PAB strains. Despite of low pH, growth of *P. acidipropionici* strains was higher than other strains. Previous studies have shown the inhibition effect of propionic acid on cell growth and propionic acid synthesis (Gu et al., 1998; Suwannakham and Yang, 2005) and some studies (Woskow and Glatz, 1991; Suwannakham and Yang, 2005; Suwannakham et al., 2006) had approached to use propionic acid-tolerant bacteria for better end results and production of metabolic end products. Thus *P. acidipropionici* strains studied in this experiment might be propionic acid-tolerant bacteria which could be promising strains for future study.

Growth and cell mass production

The addition of *p*ABA had no significant effect ($p>0.05$) on growth and cell mass production. Cereal-origin *P. acidipropionici* strains 279 and 280 reached the highest OD values. The cell mass production of dairy-origin strain 266 was the highest even though its OD was lower. It could be due to production of exopolysaccharides or biofilm, which might disturb the measurement of OD value. The mixing of the sample was difficult and might lead to lower OD value. Some PAB strains are known for their exopolysaccharides production (Gorret et al.2001).

Strain 276 failed to grow in anaerobic condition therefore it was grown in aerobic condition. Propionic acid bacteria are facultative anaerobes. The strain 276 was of cereal origin and might be used to grow with oxygen.

3.4.1.2 Folate production

Only strain 276 showed significant difference ($p < 0.05$) in folate production with two-fold increase in folate content with *p*ABA addition. The other strains did not increase their folate production with *p*ABA addition. Only in a few studies, the effect of *p*ABA on folate production has been investigated. Addition levels of 0.3mM and 1–100 µM doubled the folate production by Bifidobacteria and LAB strains, respectively (Sybesma et al. 2003; Pompie et al. 2006). Hugenschmidt et al. (2011) reported that folate synthesis in co-cultured LAB and PAB strains was 14-fold higher with *p*ABA addition than without it. They also noticed that *p*ABA supplementation above 2 mg/l did not lead to further increase in folate yields.

The PAB strains in this study might harbour all the responsible genes for folate biosynthesis which possibly could have resulted in folate production without *p*ABA addition. Some of the strains even produced folate in remarkable amount (Rossi et al. 2011; KEGG). The strains 257 and 277 produced 124 and 117 µg/l of folate, respectively, being the highest folate producers in this study. According to previous studies LAB and PAB have been observed to produce folate around 100 µg/l (Sybesma et al. 2003; Hugenholtz et al. 2002) which clearly makes strains 257 and 277 as promising folate producers and their folate production should be studied further in different cultivation conditions.

In this study, the strains excreted folate into medium from 1 ng/ml to 107 ng/ml. In the previous study by Hugenholtz et al. (2002), 41 ng/ml was the highest amount of folate in medium. Hugenschmidt et al. (2010) observed that folate excretion by PAB strains (≤14 ng/ml) was much lower than by LAB strains (≤ 397 ng/ml). Based on the previous studies, most of the PAB strains examined in this study excreted high amount of folate into medium. By contrast, strains 278 and 282 showed that they consumed folate from the medium. Strains should be cultivated in folate free medium to know the actual folate production and excretion.

In this study folate accumulation in cell biomass ranged from 3601 ng/g to 28954 ng/g cell biomass. The folate accumulation in cell biomass was lower than among yeasts (40000 ng/g – 145000 ng/g dry matter) reported by Hjortmo et al. (2005) but similar to the yeasts (10000 ng/g – 40000 ng/g cell biomass) mentioned by Kariluoto et al. (2014) and Witthöft et al. (1999). However, strains in this study showed higher folate production than some PAB strains in previous studies. Hugenholtz et al. (2002) observed that intracellular folate production by PAB strains range from 0–93 µg/l while some of the PAB strains were no able to accumulate folate inside the cell, releasing it in the medium.

3.4.2 Further studies with three selected strains

Based on their promising folate production in the screening step, strains 257, 277 and 281 were cultivated with either lactate or glucose as carbon source in PPA medium and samples were collected at four different time points (mid-exponential, early stationary, late stationary and resting phase) to examine the folate productivity and other parameters (OD, pH, cell mass).

3.4.2.1 Growth rate of strains

Strains grew faster with lactate than with glucose. Based on the growing rates, thus, time points for collecting samples in the respective growth phases were much earlier for strains cultivated with lactate as carbon source. Piveteau (1999) also noticed that lactate was utilised by PAB strains much faster than glucose. The reason for preferential utilisation of lactate is contradictory and unclear but it is probably due to shorter pathway of lactate than glucose to convert into pyruvate (Piveteau 1999).

3.4.2.2 pH, growth and cell mass production

pH of strains fermented with lactate were stable whereas pH decreased even to 4.5 for strains fermented with glucose. This was probably due to buffering capacity of lactate as was discussed in section 3.4.1.1. In earlier studies, folate biosynthesis by LAB and PAB was limited at low pH 4–5 due to cessation of growth and even cell deaths (Hettinga and Reinbold 1972; Hammes and Hertel 2009). In this study, low pH did not affect cell growth and folate synthesis which possibly caused due to aerobic fermentation condition or by acid tolerance of the strains.

OD values of the three strains were higher with glucose than lactate. Even though pH of the medium dropped, growth continued to increase. OD value increased with glucose below pH 5 as well. El-Hagarawy et al. (1957) got similar results for five *P. shermanii* strains grown on either lactate or lactose as a carbon substrate. According to Piveteau (1999), higher amounts of ATP are produced from similar amounts of sugar than from lactate which could be the reason for higher cell growth with glucose than with lactate in this study. Cell mass production was higher with glucose than lactate in strains 257 and 281 at all growth phases. By contrast only strain 277 had lower cell mass production with glucose than with lactate at stationary phases. It could be due to slow metabolism of strain with glucose.

3.4.2.3 Folate production

At the resting phase, strain 277 and 281 had the highest net folate (116 µg/l and 100 µg/l, respectively) with lactate while strain 257 had the highest net folate (113 µg/l) with glucose. This study showed that folate production depended more on individual strains than source of carbon. The level of folate production by strains was similar to folate production in screening step in anaerobic fermentation. The highest folate production by strains reached at resting phase which could be due to higher growth rate of strains leading to higher metabolic activity. Smid et al. (2001) and Sybesma et al. (2003) reported higher folate production (142 ng/ml and 200 ng/ml), respectively, by LAB strains in milk based medium. Hugenschmidt et al. (2011) reported high production of folate (8399±784 ng/ml) from LAB-PAB co-culture. 40 ng/g folate productions was reported for *Propionibacterium sp.* ABM 5378 in oat matrix by Kariluoto et al. (2014).

Intracellular folate contents with lactate were the highest at stationary phases. Strain 277 almost doubled its intracellular folate production from mid-exponential to late stationary phase. By contrast, folate excretion reduced by all three strains during stationary phase. The decrease in folate excretion in medium during stationary phases could be possibly due to consumption of folate for growth rather than synthesis of folate (Lin and Young 2000) or retention of folate inside cells due to longer glutamyl chains (Sybesma et al. 2003) rather than excreting outside in the medium. That could be the reason why intracellular folate was observed to be the highest at stationary phase.

When glucose was a carbon source, folate accumulation in cells was the highest at resting phase for strain 257 and 277 while strain 281 accumulated highest folate content during late stationary phase. The highest intracellular folate contents of the strains were almost similar regardless of growth phases. Folate excretion doubled from mid-exponential phase to resting phase but at stationary phase folate level dropped lower than initial folate amount in medium. As mentioned earlier, the possible reason could be consumption or retention of folate inside cells rather than synthesis or excretion.

When net folate production of three strains at late stationary phase in screening step was taken into consideration and compared with the results of late stationary phase in further studies, anaerobic condition with lactate showed the best folate productivity. With lactate, strains grew faster and consumed the carbon source quickly (Piveteau 1999). The growth of strains might be the factor affecting folate production as aerobic cultivation with glucose showed 2–10 folds

less folate content at stationary phases and increased later at resting phase. Net folate content of strains was lower at stationary phases than at exponential phase in further studies which is in agreement with previous study reported by Kariluoto et al. (2010) in which folate content produced by *Propionibacterium sp*. RB9 at stationary phase was lower than at exponential phase.

3.4.3 Carbon source consumption and organic acid production

The unconsumed carbon sources were measured from four growth phases. The produced amount of propionate and acetate was up to 6 g/L and 3 g/L, respectively. The production of propionate and acetate was lower than reported in studies by Bodie et al. (1987) and Schutz et al. (1987) but higher than reported in MSc thesis by Hiippala (2014). PAB strains produced from 19–30 g/L propionate and 5–10 g/L acetate (Bodie et al. 1987; Schutz et al. 1987) in milk based medium and whey medium fermented over 70 hours. Hiippala (2014) reported that the production of propionate and acetate by PAB strains less than 4 g/L and 2 g/L, respectively, when fermented in PPA medium for upto 168 hours.

In this study, all three strains had higher organic acid production with lactate than glucose. This result is in accordance with the previous studies reported by Lewis and Yang (1992a, b) where more propionate and acetate were produced during lactate fermentation than during the fermentation of sugars.

Based on the results in this study, lactate was consumed totally by all the strains. Strain 281 consumed lactate entirely before it reached the early stationary phase and glucose entirely before late stationary phase. By contrast glucose was still remained at the end of the resting phase by strain 257 and 277. The shorter metabolic pathway of lactate to pyruvate than glucose might be the factor for preferential utilisation of lactate (Piveteau 1999).

4 CONCLUSIONS

This study showed that some of the 12 screened PAB strains were promising folate producers in anaerobic condition with lactate as carbon source. The addition of *p*ABA (2mg/l) did not effect on folate production by the screened PAB strains. The strains probably either had all the genes for biosynthesis of *p*ABA or the added *p*ABA amount was too low to show effect on folate production. PAB strains produced moderate intracellular folate levels (upto 28954 ng/g cell biomass) and they also excreted folate into medium (upto 107 ng/ml). The net folate production by the best producer (strain 257) was even higher (upto 124 µg/l) than that for some LAB such as *Lactococcus spp.* and *S. thermophilus*.

When folate production by three selected strains was studied further in aerobic conditions at their four different growth phases with lactate or glucose as a carbon source, strains produced their highest net folate at resting phase with both carbon sources. It was observed that the level of folate production rather depended on individual strains than on carbon source. Although the three selected PAB strains showed their folate production capacity also in aerobic conditions, they produced even higher folate contents in anaerobic conditions. In addition, aerobic fermentation with lactate as source was observed to be the best for high organic acid production.

Because some of the screened PAB strains in this study showed their promising folate production capacity, they could be used as folate producers in food applications. However, further studies are needed to optimise the cultivation condition of selected PAB strains for their best folate production in different matrices.

5 BIBLIOGRAPHY

Ames BN. 1999. Micronutrient dediciencies cause DNA damage and cancer. Food SCi Agri Chem 1:1-15.

Arcot J and Shrestha A. 2005. Folate: methods of analysis. Trends in Food Sci and Tech. 16:253-266.

Ayad EHE. 2009. Starter culture development for improving safety and quality of Domiati cheese. Food Microbiol. 26:533–541.

Bailey LB, Rampersaud CG, Kauwell GP. 2003. Folic acid supplements and fortification affect the risk for neural tube defects, vascular diseases and cancer: evolving science. J Nutr 133(6):1961S-1968S.

Bird OD, McGlohon VM. 1972. Differential assays of folic acid in animal tissues. In: F. Kavanagh, Anal Microbiol. Vol 2. New York: Academic press. p 409-437.

Blancquaert D, Storozhenko S, Loizeay K, De Brouwer V, Viaene J, Ravanel S, Rebeille F, Lambert W, Van Der Straeten D. 2010. Folates and folic acid: from fundamental research toward sustainable health. Crit Rev Plant Sci 29:14-35.

Bodie EA, Anderson TM, Goodman N, Schwartz RD. 1987. Propionic acid fermentation of ultra-high-temperature sterilized whey using mono- and mixed-cultures. Appl. Microbiol. Biotechnol 25:434-437.

Boushey CJ, Beresford AA, Omen GS, Motulsky AG. 1996. A quantitative assessment of plasma homocysteine as a risk factor for vascular disease. J Am Med Assoc 274:1049-57.

Capozzi V, Russo P, Duenas MT, Lopez P, Spano G. 2012. Lactic acid bacteria producing B-group vitmains: a great potential for functional cereal products. Appl Microbiol Biotechnol 96:1383- 1394.

Collado MC, Meriluoto J, Salminen S. 2007a. Development of new probiotics by strain combinations: is it possible to improve the adhesion to intestinal mucus? J Dairy Res 90:2710-2716.

Collado MC, Meriluoto J, Salminen S. 2007b. In vitro analysis of probiotic strain combinations to inhibit pathogen adhesion to human intestinal mucus. Food Res Int 40:629-636.

Crittenden RG, Martinez NR, Plaune MJ. 2003. Synthesis and utilisation of folate by yoghurt starter cultures and probiotic bacteria. Int J Food Microbiol 80:217-222.

Cummins CS, Johnson JL. 1986. Genus I. Propionibacterium Orla-Jensen 1909. In: Sneath PHA, Mair NS, Sharpe ME, Holt J (eds). Bergey's manual of systematic bacteriology. Vol 2. The Williams & Wilkins Co, Balitmore. p 1346-1353.

D' Aimmo MR, Mattarelli P, Biavati B, Carlsson NG, Andlid T. 2012. The potential of bifidobacteria as a source of natural folate. J Appl Microbiol 112(5):975-984.

Daly LE, Kirke PN, Molloy A, Weir DG, Scott JM. 1995. Folate levels and neural tube defects. J Am Med Assoc 247:1698-702.

Darilmaz DO, Beyatli Y. 2012. Acid-bile, antibiotic resistance and inhibitory properties of propionibacteria isolated from Turkish traditional home-made cheeses. Anaerobe 18:122-127.

DellaPenna D. 2007. Biofortification of plant based food: Enhancing folate levels by metabolic engineering. PNAS 104:3675-3676.

De Souza S, Eitenmiller R. 1990. Effects of different enzyme treatments on extraction of total folate from various foods prior to microbiological assay and radioassay. J Micronutrient Anal. 7:37-57.

Donnelly JG. 2001. Folic acid. Crit Rev Clin Lab Sci 38: 183–223.

Edelmann M. 2014. Occurrence and natural enhancement of folate in oats and barley (Ph.D. dissertation). EKT series 1631. University of Helsinki, Department of Food and Environmental Sciences. 111-53 pp.

El-Hagarawy I, Slatter W, Harper W. 1957. Organic acid production of propionibacteria II. Effect of growth accessory substances on propionic and acetic acid production in milk. J Dairy Sci 40(6):707-12.

Eitenmiller RR and Landen WO. 1999. In: Vitamin analysis for the health and food sciences. CRC Press LLC, Boca Raton. p 411.

[FAO/WHO] Food and Agriculture Organisation of the United Nations (FAO) and World Health Organisation (WHO). 2002. Human vitamin and mineral requirements: report of a joint FAO/WHO expert consultation. Rome, Italy: FAO. p 290.

Falentin H, Deutsch S, Jan G, Loux V, Thierry A, Parayre S, Maillard M, Dherbécourt J, Cousin FJ, Jardin J, Siguier P, Couloux A, Barbe V, Vacherie B, Wincker P, Gibrat J, Gaillardin C, Lortal S. 2010. The Complete Genome of Propionibacterium freudenreichii

CIRM-BIA1T, a Hardy Actinobacterium with Food and Probiotic Applications. PLoS ONE 5(7):1-1

Finglas PM, de Meer K, Molloy A, Verhoef P, Pietrzik K,Powers HJ. 2006. Research goals for folate and related B vitamin in Europe.Eur J Clin Nutr 60. p 287–294.

Forssén KM, Jägerstad MI, Wigertz K, Witthöft CM. 2000. Folates and Dairy Products: A Critical Update. J American College of Nutr, 19:sup2. DOI: 10.1080/07315724.2000.10718071. 100S-110S.

Freisleben A, Schieberle P, Rychlik M. 2003. Specific and sensitive quantification of folate vitamers in foods by stable isotope dilution assays using high-performance liquid chromatography-tandem mass spectrometry. Anal Bioanal Chem 376:149-156.

Gangadharan D, Sivaramakrishnan, Pandey A, Nampoothir KM. 2010. Folate-producing lactic acid bacteria from cow's milk with probiotic characteristics. Int J Dairy Technol 63(3):339-348.

Gangadharan D and Nampoothiri KM. 2011. Folate production using Lactococcus lactis ssp cremoris with implications for fortification of skim milk and fruit juices. LWT - Food Sci. Technol. 44:1859–1864.

Ginting E, Arcot J. 2004. High-performance liquid chromatographic determination of naturally occurring folates during tempe preparation. J Agri Food Chem 52(26):7752-7758.

Gu Z, Glatz B, Glatz C. 1998. Effects of propionic acid on propionibacteria fermentation. Enzyme Microb. Technol 22: p 13–18.

Gorret N, Maubois JL, Engasser JM, Ghoul M. 2001. Study of the effects of temperature, pH and yeast extract on growth and exopolysaccharides production by Propionibacterium acidipropionici on milk microfiltrate using a response surface methodology. J Appl Microbiol 90(5):788-9.

Gregory JF. 1989. Chemical and nutritional aspects of folate research: analytical procedures, methods of folate synthesis, stability and bioavailability of dietary folates. Adv Food Nutr Res 33: p 1-33.

Gregory JF. 1995. In: Folates in Health and Disease. Marcel Dekker, New York, NY. pp 195.

Gregory JF. 1996 .Vitamins. In Fennema OR (ed): "Food Chemistry." New York: Marcel and Dekker. p 590–600.

Gregory JF. 1997. Bioavailability of folate. Eur J clin Nutr 51. Suppl 1: S54-9.

Gregory JF III. 2012. Accounting for differences in the bioactivity and bioavailability of vitamers. Food Nutr Res 56:10.3402/fnr.v56i0.5809.

Gujska E, Kuncewicz A. 2005. Determination of folate in some cereals and commercial cereal-grain products consumed in Poland using trienzyme extraction and high-performance liquid chromatography methods. Eur Food Res Technol 221:208-213.

Gujska E, Majewska K. 2005. Effect of baking process on added folic acid and endogenous folates stability in wheat and rye breads. Plant Food Hum Nutr 60:37-42.

Hammes WP and Hertel C. 2009. Genus I. Lactobacillus. In: P. de Vos, G. M. Garrity, D. Jones, N. R. Krieg, W. Ludwig, F. A. Rainey, K.-H. Schleifer, & W. B. Whitman (Eds.), Bergey's manual of systematic bacteriology (2nd eds).The firmicutes. Vol 3. New York, NY, USA: Springer. p 465-511.

Herranen M, Kariluoto S, Edelmann M, Pirronen V, Ahvenniemi K, Iivonen V, Salovaara H, Korhola M, 2010. Isolation and characterization of folate- producing bacteria from oat bran and rye flakes. Int J Food Microbiol 142:277-285.

Hettinga DH and Reinbold GW. (1972). Propionic-acid bacteria e review. 2. Metabolism. J Milk and Food Techno 35:358-372.

Hiippala K. 2014. The effect of different carbon sources on vitamin B_{12} production in *propionicbacterium freudenreichii* (MSc thesis). EKT series 1655. University of Helsinki, Department of Food and Environmental Sciences.

Hjortmo S, Patring J, Jastrebova J, Andlid T. 2005. Inherent biodiversity of folate content and composition in yeasts. Trends Food Sci Technol 16: 311-316.

Hjortmo S, Patring J, Andlid T. 2008a. Growth rate and medium composition strongly affect folate content in Saccharomyces cerevisiae. Int J Food Microbiol 123:93- 100.

Hjortmo S, Hellström AM, Andlid TA. 2008b. Production of folates by yeasts in Tanzanian fermented togwa. FEMS yeast Res 8:781-787.

Holasova M, Fiedlerova V, Roubal P, Pechacova M. 2004. Biosynthesis of folates by lactic acid bacteria and propionibacteria in fermented milk. Czech J Food Sci 22:175-181.

Holasova M, Fiedlerova V, Roubal P, Pechacova M. 2005. Possibility of increasing natural folate content in fermented milk products by fermentation and fruit component addition. Czech J Food Sci 23 (5): 196-201.

Holo H, Faye T, Brede DA, Nilsen T, Ødegård I, Langsrud T, Brendehaug J, Nes IF. 2002. Bacteriocins of propionic acid bacteria. Le Lait 82:59-68.

Huang Y, Adams MC. 2003. An in vitro model for investigating intestinal adhesion of potential dairy propionibacteria probiotic strains using cell line C2BBel. Lett Appl Microbiol 36:213-216.

Huang Y, Adams MC. 2004. In vitro assessment of the upper gastrointestinal tolerance of potential probiotic dairy propionibacteria. Int J Food Microbiol 91:253-260.

Hugenholtz J, Smid E. 2002. Nutrceutical production with food-grade microorganisms. Curr opin Biotechnol 13(5):497-507.

Hugenholtz J, Hunik J, Santos H, Smid E. 2002. Nutraceutical production by propionibacteria. Lait 82:103-112.

Hugenschmidt S, Schwenninger SM, Gnehm N, Lacroix C. 2010. Screening of a natural biodiversity of lactic acid and propionic acid bacteria for folate and vitamin B12 production in supplemented whey permeate. Int Dairy J 20:852-857.

Hugenschmidt S, Schwenninger SM, Lacroix C. 2011. Concurrent high production of natural folate and vitamin B12 using co-culture process with Lactobacillus plantarum SM39 and Propionibacterium freudenreichii DF13. Process Biochem 46(5):1063-1070

[IOM] Inst. Of Medicine. 2004. In: dietary reference intakes (DRIs): recommended intakes for individuals, vitamins. Food and Nutrition Board, Inst. Of Medicine. Washington, DC. Natl. Academy Press. (www.nap.edu).

Iyer R, Tomar SK. 2009. Folate: A functional food constituent. J Food Sci 74:R114-R122.

Iyer R, Tomar SK, Singh AK. 2010. Response surface optimization of the cultivation conditions and medium components for the production of folate by *Streptococcus thermophilus*. J Dairy Res 77:350-356.

Jägerstad M. 2012. Folic acid fortification prevents neural tube defects and may also reduce cancer risks. Acta Paediatr. 101:1007-1012.

Jastrebova J, Strandler H, Patring J, Wiklund T. 2011. Comparison of UPLC and HPLC for analysis of dietary folates. Chromatographia 73(5):613-613.

Kariluoto MS, Vahteristo LT, Piironen VI. 2001. Applicability of microbiological assay and affinity chromatography purification followed by high-performance liquid chromatography (HPLC) in studying folate contents in rye. J Sci Food Agric 81(9):938-942.

Kariluoto S, Vahteristo L, Salovaara H, Katina K, Liukkonen K, Piironen V. 2004. Effect of baking method and fermentation on folate content of rye and wheat breads. Cereal Chem 81(1):134-139.

Kariluoto S, Aittamaa M, Korhola M, Salovaara H, Vahteristo L, Piironen V. (2006a). Effects of yeasts and bacteria on the levels of folates in rye sourdoughs. Int J Food Microbiol 106:137–143.

Kariluoto S, Edelmann M, Herranen M, Lampi AM, Shmelev A, Salovaara H, Korhola M, Piironen V. 2010. Production of folate by bacteria isolated from oat bran. Int J Food Microbiol 143:41-47.

Kariluoto S, Minnamari Edelmann, Nyström L, Sontag-Strohm T, Salovaara H, Kivelä R, Herranen M, Korhola M, Piironen V. 2014. In situ enrichment of folate by microorganisms in beta-glucan rich oat and barley matrices. Int J Food Microbiol 176:38-48.

Keagy PM. 1985. Folacin-microbiological and animal assay. In: Augustin J, Klein BP, Venugopal PB (Eds.). Methods of vitamin assay 4th Ed. New York: Wiley. p 444-463.

Kim YI. 2004. Will mandatory folic acid fortification prevent or promote cancer? 1, 2, 3. Am J Clin Nutr 80(5): 1123-8.

Kirsch AJ and Chen TS. 1984. Comparison of conjugase treatment procedures in the microbiological assay for food folacin. J of Food Sci. 49:94-98.

Korhola M, Hakonen R, Juuti K, Edelmann M, Kariluoto S, Nyström L, Sontag-Strohm T, Piironen V. 2014. Production of folate in oat bran fermentation by yeasts isolated from barley and diverse foods. J. Appl. Microbiol. 117:679–689.

Krumdieck CL, Tamura T and Ito I. 1983. Synthesis and analysis of the pteroylpolyglutamates. Vitamins and Hormones. 40:45-104.

Laiño JE, Juarez del Valle M, Savoy de Giori G, and LeBlanc JGJ. 2013. Development of a high folate concentration yogurt naturally bio-enriched using selected lactic acid bacteria. LWT - Food Sci Technol 54:1–5.

Langer JE, Libbey LM, Day EA. 1967. Identification and evaluation of selected compounds in Swiss cheese flavour. J Agri Food Chem 15: 386-391.

LeBlanc JG, Giori GSD, Smid EJ, Hugenholtz J, Sesma F. 2007. Folate production by lactic acid bacteria and other food grade microrganisms. Comm Curr Res Edu Topics Trends Appl Microbiol 1:329-39.

Lena I. Vorobjeva, Evgeniy Yu. Khodjaev and Nina V. Vorobjeva. 2008. Propionic acid bacteria as probiotics. Microbial Ecology in Health and Disease. 20:109-112.

Lewis VP, Yang ST. 1992a. Propionic acid fermentation by *Propionibacterium acidipropionici*: effect of growth substrate. Appl. Microbiol.Biotechnol 37:437-442.

Lewis VP, Yang ST. 1992b. Continuous propionic acid fermentation by immobilized *Propionibacterium acidipropionici* in a novel packed bed bioreactor. Biotechnol Bioen 40:465-474.

Lin MY and Young CM. 2000. Folate levels in cultures of lactic acid bacteria. Int Dairy J 10: 409-413.

Liu L, Zhu Y, Li J, Wang M, Lee P, Du G, Chen J. 2012. Microbial production of propionic acid from propionibacteria: current state, challenges and perspectives. Crit Rev Biotechnol. doi:10.3109/07388551.2011.651428.

Lucock MD, Green M, Priestnall M, Daskalakis I, Levene MI, Hartley R. 1995. Optimisation of chromatographic conditions for the determination of folates in foods and biological tissues for nutritional and clinical work. Food Chem 53:329-338.

Martin JI, Landen WI, Soliman AGM and Eitenmiller RR. 1990. Application of tri-enzyme extraction for folate determination in foods. J Asso Anal Chem. 73:805-808.

Mason JB. 1995. Folate status. Effects on carcinogenesis. In: Baily LB, editor. Folate in Health and Disease. Marcel Dekker, NY. p 316-78.

McNulty H and Pentieva K. 2004. Folate bioavailability. Proc Nutr Soc 63(4):529-536.

Meile L, Le Blay G, Thierry A. 2008. Safety assessment of dairy microorganisms: propionibacterium and Bifidobacterium. Int J Food Microbiol 126: 316-320.

Morrison HI, Schaubel D, Desmeules M, Wigle DT. 1995. Serum folate and risk of fatal coronary heart disease. J Am Med Assoc 275: 1893-6.

Neta T, Takada K, Hirasawa M. 2000. Low cariogenicity of trehalose as a substrate. J Dent 28:571-576.

Newman EM and Tsai FF. 1986. Microbiological analysis of 5-methyltetrahydrofolic acid and other folates using an automatic 96-well plate reader. Anal Biochem. 154:509-515.

Nor NM, Mohamad R, Foo HL, Rahim RA. 2010. Improvement of folate biosynthesis by lactic acid bacteria using response surface methodology. Food Biotechnol 48(2):243-250.

Ohrvik VE, Witthoft CM. Human folate bioavailability. Nutrients 2011(3): 475–490. doi:10.3390/nu3040475.

Padalino M, Perez-Conesa D, Lopez-Nicolas R, Frontela-Saseta C, Ros-Berruezo G. 2012. Effect of fructooligosacharides and galactooligosaccharides on the folate production of some folate-producing bacteria in media cultures or milk. Int Dairy J 27:27-33.

Patring J, Jastrebova J, Hjortmo S, Andlid T, Jägerstad I. 2005. Development of a simplified method for the determination of folates in baker's yeast by HPLC with ultrvoilet and fluorescence detection. J Agri Food Chem 53:2406-2411.

Patring J, Jastrebova JA. 2007. Application of liquid chromatography-electrospray ionisation mass spectrometry for determination of dietary folates: Effects of buffer nature and mobile phase composition on sensitivity and selectivity. J Chromatogr A 1143:72-82.

Pawlosky RJ, Flanagan VP, Doherty RF. 2003 A mass spectrophotometric validated high-performance liquid chromatography procedure for the determination of folates in foods. J Agr Food Chem. 51(13): 3726-3730.

Piveteau P. 1999. Metabolism of lactate and sugars by dairy propionibacteria. A review. Lait 79:23-41.

Pfeiffer CM, Rogers LM, Gregory JF. 1997. Determination of folate in cereal grain food products using trienzyme extraction and combined affinity and reversed-phase liquid chromatography. J Agric Food Chem 45:407-413.

Pfeiffer CM, Fazili Z, Zhang M. 2010. Folate analytical methodology. In: Bailey LB. Folate in Health and disease. Boca Raton, FL: CRC Press. p 517-574.

Playne MJ. 1985. Propionic and butyric acids. In: M. Moo-Young (ed). Comp biotechnol. Vol 3. Pergamon press, Oxford. p 731-759.

Pompei a, Cordisco L, Amaretti A, Zanoni S, Matteuzzi D, Rossi M. 2007. Folate production by bifidobacteria as a potential probiotic property. Appl Environ Microbiol 73(1):179-185.

Poonam, Pophaly SD, Tomar SK, De S, Singh R. 2012. Multifaceted attributes of dairy propionibacteria: a review. World J Microbiol Biotechnol. Doi 10.1007/s1 1274-012-1117-z.

Rehn U, Vogensen FK, Persson SE, Hallin Saeden K, Nilsson BF, Ardo Y. 2011. Influence of microflora on texture and contents of amino acids, organic acids and volatiles

in semi-hard cheese made with DL-starter and propionibacteria. J Dairy Res 94:1098-1111.

Reisenauer AM, Krumdieck CL, Halsted CH. 1977. Folate conjugase: two separate activities in human jejunum. Science 198: 196–197.

Rolin DB, Girarad F, de Certaines JD, Boyaval P. 1995. C NMR study of lactate metabolism in Propionibacterium freudenreichii ssp. Shermanii. Appl Microbiol Biotechnol 44:210-217.

Rossi M, Amaretti A, Raimondi S. 2011. Folate production by Probiotic bacteria. A review. Nutrients 3:118-134. Doi: 10.3390/nu3010118.

Santos F, Wegkamp A, de Vos WM, Smid EJ, Hugenholtz J. 2008. High-level folate production in fermented foods by the B12 producer Lactobacillus reuteri JCM1112. Apll Environ Microbiol 74 (10):3291-3294.

Sauberlich HE, Kretsch MJ, Skala JH, Johnson HL and Taylor PC. 1987. Folate requirement and metabolism in nonpregnant women. Am J Clin Nutr 46(6):1016-1028.

Schütz M, Sievers M, Klemm K. 1987. Metabolically controlled continuous fermentation of lactose to propionate, acetate and carbon dioxide. Milchwissenschaft 42:71-74.

Selhub J and Rosenberg IH. 1981. Folate transport in isolated brush border membrane vesicles from rat intestine. J Bio Chem 236: pp 4489-4493.

Selhub J and Rosenberg IH. Folic acid. 1996. In Ziegler EE, Filer LJ Jr, eds. Present Knowledge in Nutrition. 7th ed. Washington, DC: ILSI Press: pp 206-219.

Shrestha AK, Arcot J and Paterson J. 2000. Folates assay of foods by traditional and tri-enzyme treatments using cryoprotected *Lactobacillus casei*. Food Chem. 71:545-552.

Suomalainen T, Sigvart-Mattila P, Mättö J, Tynkkynen S. 2008. In vitro and in vivo gastrointestinal survival, antibiotic susceptibility and genetic identification of *Propionibacterium freudenreichii* ssp. *Shermanii* JS. Int Dairy J 18:271-278.

Suwannakham S, Huang Y, Yang ST. 2006. Construction and characterization of ack knock-out mutants of Propionibacterium acidipropionici for enhanced propionic acid fermentation. Biotechnol. Bioeng 94: 383–395.

Suwannakham S, Yang ST. 2005. Enhanced propionic acid fermentation by Propionibacterium acidipropionici mutant obtained by adaptation in a fibrousbed bioreactor. Biotechnol Bioeng. 91: 325–337.

Swindell SR, Benson KH, Griffin HG, Renault P, Ehrlich SD, Gasson MJ. 1996. Genetic manipulation of the pathway for diacetyl metabolism in Lactococcus lactis. Appl Environ Micrbiol 62: 2641-2643.

Sybesma W, Starrenburg M, Kleerebezem M, Mierau I, de Vos WM, Hugenholtz J. 2003(a). Increased production of folate by metabolic engineering of *Lactococcus lactis*. Appl Environ Microbiol 69(6):3069-3076.

Sybesma W, Starrenburg M, Tijsseling L, Hoefnagel MHN, Hugenholtz J. 2003(b). Effects of cultivation conditions on folate production by lactic acid bacteria. Appl Environ Microbiol 69(8): 4542-4548.

Tamura T, Shin YS, Williams MA, Stokstad ELR. 1972. Lactobacillus casei response to pteroylpolyglutamates. Anal Biochem. 49:517-521.

Tamura T, Stokstad ELR. 1973. The availability of food folate in man. Bri J Haem 25. pp. 513-532.

Tamura T. 1998. Determination of food folate. J Nutr Biochem 9: 285–293.

Thierry A, Maillard MB. 2002. Production of cheese flavour compounds derived from amino acid catabolism by *Propionibacterium freudenrichii*. Le Lait 82:17-32.

Thierry A, Maillard MB, Bonnarme P, Roussel E. 2005. The addition of *Propionibacterium freudenrichii* to Raclette cheese induces biochemical changes and enhances flavour development. J Agric Food Chem 53:4157-4165.

Tomar SK, Srivatsa N, Iyer R, Singh R. 2009. Estimation of folate production by Strptococcus thermophiles using modified microbiological assay. J Milchwissenschaft 64(3):206-263.

Uchida M, Yoda N, Terahara M, Seki K, Choi SSH, Roberts A. 2011. Safety evaluation of *Propionibacterium freudenrichii* ET-3 culture. Regul Toxicol Pharmacol 60:249-261.

Vahteristo LT, Ollilainen V, Pekka EK, Varo P. 1996. Improvements in the analysis of folic acid by high-performance liquid chromatography. J Agri and Food Chem. 44:477-482.

Vahteristo LT, Ollilainen V, Varo P. 1997. Liquid chromatographic determination of folate monoglutamates in fish, meat, egg and dairy products consumed in Finland. J AOAC Int 80:337-8.

Van Wyk J, Witthuhn RC, Britz TJ. 2011. Optimisation of vitamin B12 and folate production by *Propionibacterium freudenreichii* strains in Kefir. Int Dairy J 21(2): 69-74.

Vishnumohan S, Arcot J, Pickford R. 2011. Naturally-occuring folates in foods: Method development and analysis using liquid chromatography- tanden mass spectrometry (LC-MS/MS). Food Chem 125(2). pp. 736-742.

Weber W, Mönch S, Rychilk M, Stengl S. 2011. Quantitation of vitamins using microbiological assays in microtiter formats. In: Rychilk M. Fortified foods with vitamins. Weinheim, Germany: Wiley-VCH Verlag & Co. pp. 37-75.

Wegkamp A, van Oorschot W, de Vos WM, Smid EJ. 2007. Characterisation of the role of *para*-aminobenzoic acid biosynthesis in folate production by *Lactococcus lactis*. Appl Env Microbiol 73(8):2673-2681.

Witthöft C, Forssen K, Johannesson L, Jägerstad M. 1999. Folates- food sources, analyses, retention and bioavailability. Scand J Nutr 43:138-146.

Woskow SA and Glatz BA. 1991. Propionic acid production by a Propionic Acid-Tolerant Strain of *Propionibacterium acidipropionici* in batch and semicontinuous fermentation. Appl Env Microbiol 57(10). pp 2821-2828.

Zárate G, Chaia AP. 2012. Influence of lactose and lactate on growth and beta-galactosidase activity of potential probiotic *Propionibacterium acidipropionici*. Anaerobe 18:25-30.

Zárate G, Chaia AP, Oliver G. 2002. Some characteristics of practical relevance of the beta-galactosidase from potential probiotic strains of *Propionibacterium acidipropionici*. Anaerobe 8:259-267.

APPENDIX 1 Calculation used to determine glucose concentration

Glucose concentration equivalent to 60% sodium lactate

Moles= mass/molar mass

M=60*1000/100*112.6

M= 5.328 mol/l and 0.0745 moles in 14 ml sodium lactate

Half a mole of glucose equivalent to 1 mole of lactate

0.075/2= 0.0375 moles of glucose per litre

Mass= Moles * molar mass

Mass= 0.0375 mol * 180.16 g/mol =6.756 g/l (0.67% w/v)

APPENDIX 2 Composition for preparation of Extraction buffer

Ingredients for 150 ml volume of water

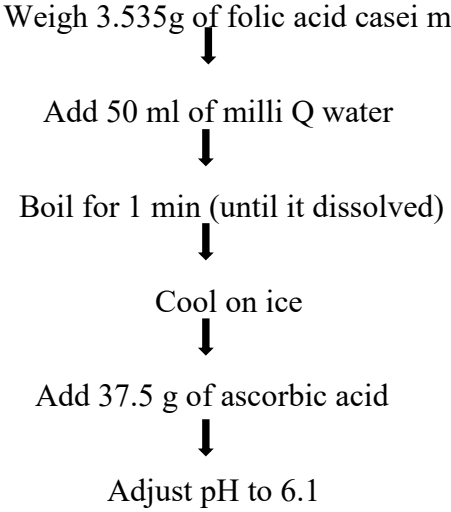
Hepes- 1,788g

Ches- 1,554g

Sodium ascorbate- 3 g

2-mercaptoethanol- 105 µl

APPENDIX 3 Flow chart of preparation of assay medium



APPENDIX 4 Statistical analysis of OD, pH, cell mass and folate

Table 1. Statistical analysis of OD, pH, cell mass and folate. Variables were tested using two-tailed Independent test. A p-value of < 0.05 was considered statistically significant.

		With *p*ABA			
	Strain	OD	pH	Cell mass	folate
Without *p*ABA	257	0.55	0.091	0.479	0.071
	259	0.872	0.181	0.538	0.916
	264	0.932	0.643	0.762	0.473
	266	0.647	0.678	0.869	0.121
	275	0.252	0.507	0.8	0.489
	276	0.177	0.524	0.05	0.04*
	277	0.183	0.417	0.318	0.348
	278	0.478	1	0.634	0.666
	279	0.289	0.035*	0.78	0.621
	280	0.012*	0.768	0.064	0.449
	281	0.094	0.815	0.049*	0.551
	282	0.868	0.035*	0.455	0.888